Resistance to CD40-Mediated Sickness Behavior Syndrome by Reprogramming of Tissue Macrophages and Monocytes

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1. ZUSAMMENFASSUNG

Das Sickness Behavior Syndrome (SBS) umfasst die verschiedenen, durch das Immunsystem vermittelten Anpassungen von Verhalten und Physiologie die zur Bekämpfung von Infektionen und Erholung von Verletzungen beitragen. Zu diesen Verhaltensänderungen gehört unter Anderem ein erhöhter Schlafdruck zusammen mit fragmentiertem Schlaf, Erschöpfung, Appetitsverlust, sowie Niedergeschlagenheit und sozialem Rückzug. Im Kontext von chronischen Erkrankungen sind diese Verhaltensänderungen jedoch unangebracht und tragen wesentlich zur Verringerung der Lebensqualität der Patienten bei, oft mehr als die Symptome der zugrunde liegenden Erkrankung. Zytokine, Botenstoffe des Immunsystems die, neben anderen Funktionen, dem Gehirn den Status einer Entzündung oder Verletzung übermitteln, lösen Änderungen in der Aktivität bestimmter Hirnregionen aus, welche in den erwähnten Symptomen resultieren. Pro-inflammatorische Zytokine wie Tumornekrosefaktor (TNF) oder Interleukin-1β (IL-1β) und anti-inflammatorische Zytokine wie IL-10 haben hierbei gegensätzliche Effekte.

Wir zeigen hier, dass die Produktion von Zytokinen durch Makrophagen der entscheidende Mechanismus ist, welcher der Entstehung des Sickness Behavior Syndroms zugrunde liegt. Unter Bedingungen hoher, systemischer Aktivierung des CD40 Signalwegs, was bei Autoimmunerkrankungen häufig der Fall ist, können inflammatorische Monozyten durch Blockierung des Rezeptors für den Koloniestimulierenden Faktor 1 von einem pro- zu einem anti-inflammatorischen Phänotyp umprogrammiert werden. Die darauf folgende Ausschüttung grosser Mengen IL-10 wirkt der Schlaf-induzierenden und Anorexie-verursachenden Wirkung von TNF und anderer pro-inflammatorischen Zytokinen entgegen. Dies resultiert in einem nahezu völligen Schutz vor den häufigsten Symptomen des SBS, Schläfrigkeit und Kachexie.

Zytokine haben ausserdem Einfluss auf die zirkadiane Rhythmik indem sie die Gene der molekularen Uhr (Clock Gene) herunterregulieren. Wichtige Funktion der molekularen Uhr, wie die Festlegung von Zeitverlauf und Konsolidierung von Schlaf-Wach Rhythmen, die Regulierung des Metabolismus entsprechend der Notwendigkeiten des Tagesrhythmus, und die Modulierung der Reaktionsfähigkeit

des Immunsystems, gehen verloren. Dies geschieht vermutlich um eine durchgehende Entzündungsreaktion und Verhaltensanpassung zu ermöglichen.

Mit dem Kälte-induzierbaren RNA-bindenden Protein (*Cirbp*), welches die mRNA verschiedener Clock Gene stabilisiert, identifizierten wir einen neuen Mechanismus der Clock Gen Regulation durch Zytokine. Die Expression von Cirbp wird durch TNF stark herunterreguliert, was wiederum zu einer deutlichen Reduktion der Expression mehrerer Clock Gene führt.

1. SUMMARY

The sickness behavior syndrome (SBS) comprises the collective immune-mediated adaptations of behavior and physiology that help to facilitate recovery from infection or injury. These adaptations include increased sleep propensity in combination with sleep fragmentation, fatigue, loss of appetite, as well as lowered mood and social withdrawal. In chronic diseases however, these otherwise useful behavioral changes become maladaptive and severely affect the quality of life of patients, often times more than the symptoms of the underlying disease. Cytokines, messenger molecules of the immune system that, among other functions, signal the state of infection or injury to the brain, elicit changes in neural activity, which results in the aforementioned symptoms. Proinflammatory cytokines like tumor necrosis factor (TNF) and interleukin-1β (IL-1β), and anti-inflammatory cytokines like IL-10 have antagonistic functions in this regard.

Here we show that macrophages are the key regulators of the sickness behavior response, especially in the context of autoimmune diseases. Under conditions of high CD40 signaling, which is common in autoimmune diseases, inflammatory monocytes can be switched from a pro- to an anti-inflammatory phenotype, by neutralizing the receptor for colony stimulating factor 1. The following production of high amounts of IL-10 counteracts the somnogenic and anorexigenic effects of TNF and other proinflammatory cytokines, resulting in nearly complete protection from the most common symptoms of SBS, daytime sleepiness and cachexia.

Cytokines also affect circadian rhythmicity through a profound downregulation of clock genes. Important functions of the molecular clock, like timing and consolidation of sleep-wake patterns, regulation of metabolism according to the daily cycle, and immune modulation are lost. The most likely reason for this is to enable a sustained inflammatory and behavioral response. With the cold inducible RNA-binding protein (CIRBP), a chaperone that stabilizes the mRNA of several clock genes, we were able to identify a novel mechanism of cytokine-mediated clock gene regulation. Expression of *Cirbp* is strongly downregulated after challenge with TNF, resulting in a marked reduction in the expression of several clock genes and clock-controlled genes.

2. INTRODUCTION

2.1 The immune system

The vertebrate immune system is a highly complex network of cellular and humoral components that act in concert to defend the organism from pathogens and toxins, mediate tissue repair in case of injury, and help in maintaining metabolic and tissue homeostasis. Regulation of these functions is achieved through compartmentalized cell-to-cell signaling as well as the action of signaling peptides and proteins such as chemokines and cytokines. The defense against pathogens in higher vertebrates is organized in three distinct layers that are continuously active and act in concert. The first layer consists of physical barriers like the skin or epithelial mucosa, which prevent direct entry of microorganisms into the underlying tissue. The next layer in host defense comprises the phagocytic and cytotoxic cells of the so-called innate immune system. They recognize microorganisms and damaged, infected or malignant cells through conserved pattern recognition receptors (PRRs) on their cell surface. This results in the production of cytokines, chemokines, and, depending on the stimulus, either in phagocytosis or the release of toxic molecules. Although the innate immune system is a fast (hours) and effective layer of protection, it may not be sufficient to clear a given infection. In this case, an adaptive immune response is initiated. The adaptive immune system consists of B and T cells, which have specialized pattern receptors that are randomly generated during their development and are unique for each cell. Thus, each single cell recognizes only one specific target structure termed antigen (as in 'antibody generating'), and the system as a whole has nearly unlimited recognition potential. Only those cells that recognize an invading pathogen will take part in the immune response against it, hence the term 'adaptive immunity'. These three different layers or arms of the immune system are highly conserved among vertebrates and represent the evolutionary pathway through which our immune system has developed (Rodríguez et al., 2012).

The process that accompanies an acute immune response is called inflammation, during which the immune system regulates blood flow and vascular permeability to recruit phagocytic and other immune effector cells into the affected tissue. This typically results in swelling, redness, and locally elevated temperature. Increased

sensitivity to pain induces protective behavior that helps to prevent further contact with pathogens and supports wound healing by resting of the injured body part. Together with these basic functions, the immune system also modifies behavior and physiology as part of a highly organized, adaptive strategy that is collectively termed sickness behavior syndrome (SBS). It comprises such symptoms as fever, fatigue and sleepiness, loss of appetite, anhedonia and lowered mood paired with reduced social activities (Dantzer and Kelley, 2007). The increased body temperature during fever stimulates immune responses and limits the proliferation of thermo-sensitive pathogens (Kent et al., 1992). Fatigue, increased sleep and social withdrawal limit the spread of pathogens between members of a population and help to conserve energy, which, in the context of reduced food uptake, is a crucial survival mechanism. Aside from obvious regenerative purposes, sleep seems to also play an important role in the generation of fever (Imeri and Opp, 2009). Lastly, increased pain sensitivity limits the risk of further injury. Taken together, these adaptations offer a universal selective advantage for individuals and populations as a whole, and are therefore common for most diseases. In the context of chronic inflammatory diseases caused by infectious pathogens and autoimmune reactions, however, this strategy becomes maladaptive, and patients suffering from such conditions often cite the accompanying symptoms of SBS as more encumbering, and more difficult to cope with, than the actual symptoms arising due to organ dysfunction. Moreover, uncontrolled activation of the immune system with overproduction of cytokines has been linked to the pathogenesis of major depressive disorder (Dantzer et al., 2011; Miller et al., 2009).

2.1.1 Immune signaling to the brain

In the early 1960s, studies with bacterial lipopolysaccharide (LPS) in rats suggested that SBS correlates with the presence of soluble inflammatory mediators in the blood, a finding that was later confirmed for interleukin (IL)-1β, tumor necrosis factor (TNF) and other cytokines (Holmes and Miller, 1963; Kent et al., 1992; Toth and Opp, 2001). When administered peripherally or directly into the cerebral ventricles, cytokines including TNF, IL-1β, IL-6, and interferons (IFN) induce SBS. Likewise, SBS following stimulation of Toll-like receptor (TLR) 3 with synthetic double-stranded RNA (polyI:C)

or TLR4 with LPS, is associated with an increase in cytokine production both in the central nervous system (CNS) and in the immune compartment (Bluthé et al., 2000; Cunningham et al., 2007).

The general function of cytokines is to transmit information about the type of pathogen and state of infection or injury to the appropriate target cells of the immune system. Proinflammatory cytokines induce proliferation, maturation, apoptosis, and the production of effector molecules, while anti-inflammatory cytokines have generally opposite functions. The complex network of these and other signaling molecules and their respective receptors orchestrates the immune response according to the state of infection or injury. Having evolved more than 850 million years ago, cytokines are evolutionary ancient molecules and highly conserved in both vertebrates and invertebrates. This, as well as the presence of cytokines and their receptors in the brain in the absence of an immune challenge, suggests that they originally may have had non-immune functions, explaining their highly complex dual role in host protection (Opp, 2005). The central nervous system possesses a specialized environment and is shielded from circulating molecules, including cytokines, by tight junctions, pericyte coverage, and blood-directed vesicle trafficking in endothelial cells. Together, these elements of the CNS vasculature function as the so called blood-brain barrier (BBB). Signal transmission across this barrier is highly regulated and can occur by a number of different mechanisms and specialized structures.

For example, the first discovery in this regard was the ability of vagal nerve endings to detect the presence of cytokines in the periphery and transmit this information to their target structures in the CNS (Bluthé et al., 1996). Such signaling can evoke a multitude of behavioral responses, mediated at least in part by the local release of cytokines in the brain (Gaykema et al., 2000; Laye et al., 1994).

Circumventricular organs (CVOs) are specialized brain structures at the interface of ventricles and brain parenchyma that do not possess a conventional BBB. Their fenestrated blood vessels and lack of tight junctions allows the diffusion of substances that would normally be prevented from entering (or leaving) the brain (Morita and Miyata, 2012). CVOs are either secretory, enabling neurons to release a variety of peptide hormones into the blood stream, or sensory, which detect plasma molecules, including cytokines, and convey this information to other brain regions for the control

of autonomic and inflammatory reactions and behaviors (Morita and Miyata, 2012). The latter include the area postrema, the subfornical organ (SFO) and the organum vasculosum of the lamina terminalis (OVLT). While blood-derived molecules move freely within the parenchyma of the CVOs, they do not diffuse into the neighboring tissue, indicating a functional BBB that segregates the CVOs from the rest of the brain (Fry et al., 2006).

Signals may also be transduced by endothelial, most of which express cytokine receptors and respond to them, for example by producing inflammatory mediators such as prostaglandins and NO (D'Mello and Swain, 2011). Furthermore, many cytokines have been reported to be transported across the BBB, including TNF, IL-1β and IL-6, for which specific unidirectional transporters are postulated to exist (Banks et al., 1991; 1994; Gutierrez et al., 1993). However, the latter studies were performed by only one group of researchers, and by using only a single method approach. These data may therefore be subject to several confounding factors, and should be considered unconfirmed.

2.1.2 Autoimmune diseases

A common trait of many autoimmune diseases is the upregulation of the co-stimulatory molecule CD40L (CD154) by $CD4^+$ T cells. This member of the TNF superfamily has first been described as strong inducer of B cell proliferation in vitro when given together with IL-4 and other cytokines. Its cognate receptor, CD40, is expressed on B cells, macrophages, monocytes, microglia, dendritic cells (DCs), natural killer (NK) cells, as well as non-immune cells like endothelial and epithelial cells, fibroblasts and myofibroblasts. In many models of T cell-mediated autoimmunity in mice, for example in experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis or colitis, development of autoimmunity can be prevented by blocking CD40 - CD40L signaling (De Jong et al., 2000; Durie et al., 1993; Howard et al., 1999). In case of EAE, progression of the disease is dependent on CD40 in the microglial compartment (Becher et al., 2001). From a different perspective, agonistic antibodies to CD40 or soluble CD40L have therapeutic potential in the treatment of lymphoproliferative malignancies and solid tumors, but their applicability is limited

due to the occurrence of SBS with fatigue, anorexia and headache (Advani et al., 2009; Furman et al., 2010; Hussein et al., 2010; Wierda et al., 2000).

As part of their immune surveillance function, antigen-presenting cells (APCs), including macrophages, DCs and B cells, continually take up antigen in the periphery and subsequently migrate to secondary lymphoid organs where they encounter naïve T cells. Upon recognition of a foreign antigen through T cell receptor binding of MHC II molecules, $CD4^+$ T cells readily express CD40L on their cell membrane. This in turn leads to the maturation of the APC, resulting in cell proliferation, increased stability of major histocompatibility class II (MHC II) complexes, and the production of proinflammatory cytokines including IFNy, IL-6, and TNF. In case of B cells, binding of CD40 is also required for isotype switching (as demonstrated by the X-linked hyper IgM syndrome) as well as germinal center formation and affinity maturation. Since DCs do not mature properly if CD40 signaling is blocked, the differentiation of inflammatory T cells and the production of IL-12 are also strongly impaired. Thus, CD40/CD40L signaling is essential for the activation of B cells and APCs, and is a critical factor in the development of both B cell and T cell dependent adaptive immune responses. Mechanistically, crosslinking of CD40 through CD40L or via binding of a monoclonal antibody induces recruitment of different TRAF signaling complexes to the plasma membrane, culminating in the activation of nuclear factor κB (NFκB), c-Jun N-terminal kinase (JNK) and protein kinase B (PKB/Akt). An alternative pathway through Janus kinase (Jak) 3 has been described for macrophages and DCs. The same effect can be achieved by administration of an agonistic monoclonal antibody against CD40 (Danese et al., 2004; Elgueta et al., 2009; Grewal and Flavell, 1998).

Antibody-mediated CD40 ligation was introduced recently as a new model system for the induction of sickness behavior (Cavadini et al., 2007). The effect of CD40 antibodies on behavior relies on TNF/TNFR1 signaling (Taraborrelli et al., 2011). TNF, like CD40, has been linked to the pathology of many rheumatic and autoimmune diseases, and the use of anti-TNF agents has become routine clinical practice in the treatment of autoimmune diseases, including rheumatoid arthritis and psoriasis (Feldmann and Maini, 2003; Kassiotis and Kollias, 2001). Some studies have indicated a dual role for TNF in the progression of autoimmune diseases, however. For example, TNF deficient mice develop severe autoimmune arthritis following immunization with collagen.

Similarly, TNF knockout animals are not protected from the development of EAE, although disease onset is consistently delayed (Eugster et al., 1999; Kollias et al., 1999). The common theme seems to be that while TNF is an important factor for disease induction in these models, it may also have immunosuppressive functions during later stages of autoimmune diseases (Cope, 1998). Understanding the link between CD40 and TNF as well as their effects on behavior and immune regulation may therefore open up new avenues for disease intervention.

2.1.3 Macrophages

Macrophages comprise a central part of the innate immune response and are at the forefront of host defense. They are widely distributed through nearly all tissues, where they act as scavengers that can quickly detect invading pathogens and tissue injury through expression of a wide variety of pattern recognition receptors (PRRs). During steady state conditions, numbers of tissue resident macrophages are replenished by local proliferation, whereas during inflammation, circulating monocytes are recruited to reinforce and replace the local cell pool (Hashimoto et al., 2013). These inflammatory monocytes, which are characterized by their high expression of the leukocyte marker Ly6C, not only give rise to macrophages or DCs, but also contribute to immune defense by secreting proinflammatory cytokines and anti-microbial factors (Serbina et al., 2007). Like DCs, albeit less efficiently, macrophages are continuously sampling their environment via receptor-mediated phagocytosis and present the processed antigens to T cells through MHC II molecules. Upon contact with pathogen- or damage associated patterns (PAMP or DAMP respectively), macrophages enter an 'activated' state, which is characterized by increased phagocytic and antigen-presenting capabilities as well as the production of proinflammatory cytokines and chemokines like TNF, IL-1β and CCL2. Through these mechanisms, macrophages alert surrounding immune cells that have not yet encountered antigen, and direct the influx of neutrophils and monocytes into the affected tissue. After taking up antigen, macrophages migrate to secondary lymphoid organs where they participate in the induction of the adaptive immune response. After clearance of pathogens and cell debris from the site of inflammation, macrophages can undergo a phenotypic shift that results in the production of

proinflammatory cytokines like IL-10 and TGF-β, leading to the resolution of the immune response (Mosser, 2003). Depending on the type of pathogen, bacterial and viral versus extracellular parasites like helminths or protozoans, macrophages can assume different stages of activation. Historically, these different functional states have been termed as classically or alternatively activated. The division into these two groups has been proven to be insufficient however, as macrophages have many other functions aside from host defense, such as clearance of dead or apoptotic cells, tissue regeneration and angiogenesis. Many tissue resident macrophages have specialized functions in tissue homeostasis. For example, macrophages in bone marrow and fetal liver are essential for the development of erythrocytes, while red pulp macrophages and Kupffer cells remove old and non-functional erythrocytes from the circulation (de Back et al., 2014; Kawane et al., 2001). Osteoclasts on the surface of the bone degrade bone matrix and act as counterpart to the continuous bone formation by osteoblasts. Microglia, the tissue macrophage-like cell in the CNS, interacts with neurons and astrocytes and are thereby involved in signaling pathways of the neuronal network. Taken together, macrophages encompass a wide variety of functions, ranging from pathogen clearance and immune regulation, to wound healing and both tissue and metabolic homeostasis.

Several classification schemes have been proposed, and the most recent one defines classically activated macrophages as M1 and the group of alternatively activated macrophages as M2a-c (Martinez et al., 2009; Mills et al., 2000). However, macrophages and cells of the monocyte lineage in general, seem to exist in a continuum of different functional states that are fine tuned by the local microenvironment (Mosser and Edwards, 2008). It is for this reason that the division into distinct phenotypes of M1, M2 and subtypes thereof is not clear-cut, with cells having overlapping functional characteristics. Consequently, a new classification system needs to be developed, one that may be based, for example, on gene expression profiles. Unlike lymphocytes, where phenotypic changes are 'fixed' by chromatin modifications after exposure to polarizing cytokines, macrophages have a highly dynamic gene expression profile, which is influenced by the type, the concentration, and the timing of exposure to stimulating agents in the microenvironment (Cassetta et al., 2011; Mosser and Edwards, 2008). This is accompanied by the ability of macrophages to revert to their

original functional state after cytokine signaling ceases (Häusser et al., 1997; Stout, 2004). The extraordinary plasticity of macrophages is the basis of their functional heterogeneity during the immune response, during development, and in tissue homeostasis.

2.2 Sleep in health and disease

Sleep is a complex physiological process putatively serving several biological functions including energy conservation, tissue regeneration and memory consolidation (Cirelli, 2009; Siegel, 2005). It can be subdivided into different phases according to characteristic differences in electroencephalogram (EEG) activity. There are two types of sleep in general, which follow each other during the 4 to 5 sleep cycles typical for humans. Non rapid eye movement (NREM) sleep is the most prominent sleep phase and can be divided into four separate stages: a transitional stage 1, followed by stage 2 were wake associated alpha waves (8-13 Hz) disappear. Stage 3 and 4 are dominated by slow (1-4 Hz), high voltage oscillations, termed 'slow' or 'delta' waves. This 'slow wave sleep' (SWS) is generally considered as restorative sleep were energy stocks are replenished and synaptic connections are downscaled depending on prior use. During this stage, neurons fire in bursts followed by a short period of inactivity. Bursts are synchronized throughout the cortex by hypothalamic activity, resulting in the slow, high voltage spiking patterns observed in the EEG. The intensity of NREM sleep is determined by the prevalence of these slow waves as assessed by fast Fourier transform (FFT) analysis, and usually expressed as slow wave activity (SWA) or 'delta power'.

Rapid eye movement (REM) sleep on the other hand does not possess an intensity dimension. It is characterized by fast (4-7 Hz), low voltage oscillations (theta waves), which are similar to those seen during wakefulness. It represents the time during which dreams are experienced, the function of which is still unclear, as is the case for REM sleep per se.

In 1982, Borbély and colleagues introduced a mathematical model that made it possible to predict sleep need in humans and other mammals as expressed by the prevalence of EEG slow wave activity during sleep (Borbély, 1982; Borbély and Achermann, 1999). It consists of a homeostatic component termed Process S, which predicts changes in SWA

as a function of the sleep-wake history, and a circadian oscillator termed Process C, which determines periods of wakefulness and sleep in the context of daily light-dark cycles. During the day, this circadian clock opposes the accumulating sleep pressure to keep the organism awake, while during the night, it consolidates sleep as sleep pressure is continually reduced.

2.2.1 Circadian and homeostatic regulation of sleep

The accumulation of adenosine during prolonged wakefulness as byproduct of ATP degradation has been proposed as a major homeostatic mechanism for sleep induction. Adenosine decreases neuronal activity by binding to adenosine receptors in the brain (Bjorness and Greene, 2009; Porkka-Heiskanen et al., 2000; Radulovacki et al., 1984). In addition, ATP was shown to act via purine receptors on glia to release IL-1β and TNF, which may in turn induce local changes in neuronal networks that a similar to those seen in sleep (Krueger, 2008; Vyazovskiy et al., 2011). Other sleep regulatory substances include growth hormone releasing hormone (GRHR) and brain-derived neurotrophic factor (BDNF) for NREM, and prolactin and nitric oxide (NO) for REM sleep (Huber et al., 2007b; Krueger, 2008). The physiological basis of Process C is given by a molecular clock that is active in almost all cells and is entrained to the light-dark cycle through a central pacemaker in the suprachiasmatic nucleus (SCN) (Hastings et al., 2003; Landgraf et al., 2011). The interaction between these processes accounts for essential aspects of sleep regulation. Some adaptations have been made to the two-process model since its original conception. For example, dissipation of SWA is not uniform but localized in different cortical areas. To accommodate for these regional changes, a Process Z was proposed (Zavada et al., 2009).

2.2.2 Brain structures involved in sleep regulation

While the two-process model enables us to study sleep homeostasis, it does not explain the neuroanatomical basis for sleep. In 1917, Austrian neurologist Constantin von Economo began to see patients with a new type of encephalitis that resulted in patients sleeping for more than 20 hours daily, only waking briefly to eat and drink. The disease was eventually named encephalitis lethargica, or sleepy sickness, and after roughly a

decade, it disappeared with only isolated cases being reported since. While the definite cause of the disease was never identified, von Economo could determine the brain regions in which lesions caused the alteration of the sleep-wake cycle. Since all patients had lesions at the junction of the midbrain and the diencephalon, he concluded that there was an ascending arousal system originating in the brain stem, which kept the forebrain awake. Studies during the second half of the last century have demonstrated the existence of monoaminergic and cholinergic nuclei projecting from the reticular formation in the rostral pons to the thalamus, the lateral hypothalamus and to the cerebral cortex (Lindsley et al., 1949; Moruzzi and Magoun, 1949). Thus, the concept of the ascending reticular activating system (RAS) was developed (Saper et al., 2005). The sleep-promoting counterpart of the RAS consists of the GABA-ergic ventrolateral preoptic nucleus (VLPO), which in turn is driven by the median preoptic nucleus (MnPO). Both are selectively active during sleep and suppress the arousal system (Sherin et al., 1998; Takahashi et al., 2009; Uschakov et al., 2007). Experiments have shown that lesions in this region reduce REM and NREM sleep by more than 50% (Lu et al., 2000). These sleep- and wake-promoting neuronal systems are mutually inhibiting, forming a 'flip-flop' switch that enables sharp transitions between sleep and wakefulness, while avoiding transitional states (Saper et al., 2010).

2.2.3 Cytokines and sleep

A multitude of information including day length, nutritional status, external temperature, as well as input from limbic and cortical areas is integrated to regulate the state of arousal. During disease, cytokines can override circadian and homeostatic mechanisms of sleep-wake regulation to enforce behavioral responses beneficial to recovery. Many cytokines have been shown to increase NREM sleep and EEG delta activity after systemic or central administration (Zielinski and Krueger, 2011). Among these, IL-1β and TNF are best described. They were shown to have a direct effect on neuronal activity by binding to their respective receptors on neurons and glia cells.
IL-1β is one of 11 members of the interleukin 1 family of cytokines. Other sleep altering members include the proinflammatory IL-1α and IL-18, which increase NREM sleep, and the proinflammatory IL-1Ra, which reduces NREM sleep. Due to the homology of

its cytoplasmic domain with those of Toll-like receptors (TLR), signaling through IL-1 receptor 1 and 2 shares many inflammatory functions with this group of innate immune receptors, including induction of cyclooxygenase type 2 (COX2), type 2 phospholipase A, inducible nitric oxide synthase (iNOS), and increased expression of cellular adhesion molecules. As a result, IL-1β induces fever, hyperalgesia, vasodilatation, and directs the influx of immune cells into inflamed tissues (Dinarello, 2009). Through its induction of IL-6 and IL-23, IL-1β is an essential factor for the differentiation of T_h17 cells.

TNF is part of a large family of evolutionary conserved signaling molecules. It was first discovered as a factor mediating tumor control and cachexia, and later as an endogenous pyrogen (Zielinski and Krueger, 2011). Subsequent studies have demonstrated the role of TNF as a central molecule in innate immunity possessing a broad range of immune regulatory functions. Depending on cell type and environment, TNF can induce apoptosis, cell proliferation and differentiation, as well as the expression of cytokines, chemokines and other inflammatory mediators (Vilcek and Lee, 1991). Two ubiquitously expressed cell-surface receptors for TNF have been described, TNF receptor 1 and 2 (TNFR1 and TNFR2), which have non-redundant functions (Chu, 2013).

While the antimicrobial and inflammatory actions of the immune system are necessary in the defense against pathogens, they also cause damage to host cells and tissues. As seen in septic shock, an uncontrolled inflammatory response caused by excessive cytokine production may even result in death. Therefore, mechanisms that keep the immune system in check and terminate cytokine production when appropriate are an essential component of the immune response.

The anti-inflammatory cytokine IL-10, for example, has long been recognized as an important immune regulatory molecule. It is produced mostly by monocytes/macrophages and regulatory T and B cells and has pleiotropic functions that are highly context dependent. The IL-10 receptor is a tetrameric complex consisting of two molecules of IL-10 receptor 1 and 2 each. Its expression is induced during infection or injury in many different tissues and cell types, including cells of the hematopoietic lineage, endothelial cells, cells of the gastric epithelium, synovial fibroblasts, as well as glial cells and neurons (Gonzalez et al., 2009; Moore et al., 2001;

Sharma et al., 2011). IL10 signaling inhibits the production of proinflammatory cytokines and induces the downregulation of MHC II proteins and co-stimulatory molecules on the surface of APCs, reducing their capability of priming and perpetuating an adaptive immune response. In this way, IL-10 regulates the important balance between host defense and prevention of immune-mediated damage. The failure of keeping sufficient IL-10 signaling seems to be the underlying basis for many neuroimmune pathologies such as multiple sclerosis or neuropathic pain, and injection of recombinant IL-10 is emerging as a valuable tool in the treatment of such conditions (Groux and Cottrez, 2003; Kwilasz et al., 2014). Moreover, IL-10 is an important factor in maintaining peripheral tolerance to commensal bacteria in the gut, a process that involves it's production by regulatory T cells. Mice lacking either IL-10 or IL-10 receptors spontaneously develop inflammatory bowel disease (Leach et al., 1999).

Next to its anti-inflammatory functions, IL-10 has also been shown to stimulate B cells and mast cells, and plays a role in the development of T_h2 responses (Rousset et al., 1992; Thompson-Snipes et al., 1991).

There are numerous observations implicating a role of cytokines in the physiological regulation of sleep. For example, TNF expression and protein levels in the brain are increased by continued wakefulness. Moreover, TNF levels in rats are highest at the point of sleep onset, and decline during the sleep phase (Krueger, 2008). TNF receptor 1 was shown to affect sleep regulation, as it's knockout reduces sleep during the resting period and dampens the effect of sleep deprivation and sleep fragmentation (Deboer et al., 2002; Ramesh et al., 2012).

Another line of evidence comes from direct injections of cytokines into the CNS. When either TNF or IL-1β is applied to single columns or unilaterally on the cortex, only the cortical column or hemisphere in direct contact with the respective cytokine will show an increase in NREM activity. This points to a strictly para- and/or autocrine function of these substances in sleep regulation. Moreover, a study in rats has shown that during prolonged wakefulness, groups of cortical neurons will begin to briefly stop firing, as in sleep, leading to localized EEG slow wave activity. This phenomenon of 'local sleep' increases with the time the animal spends awake, and is accompanied by progressive cognitive impairment (Vyazovskiy et al., 2011).

IL-10 has been shown to reduce spontaneous sleep when injected intracerebroventricular, thus mirroring its functional antagonism to TNF and IL-1β in immune responses (Kushikata et al., 1999; Opp et al., 1995). Whether this effect is due to a downregulation of TNF and IL-1β or because of a direct effect of IL-10 on neurons, is still unclear.

Taken together, these findings suggest that sleep is a use-dependent phenomenon mediated by local release of sleep-inducing substances, and synchronized throughout the brain by sleep-regulatory circuits.

2.3 *Circadian rhythms*

The Earth's 24h cycle of day and night, along with the concomitant changes in temperature, imposes on organisms the need to adapt to these continuous changes in environmental conditions. The development of light sensing molecules, together with a molecular clock mechanism, enabled organisms to anticipate these changes and align their metabolism and behavior accordingly. First evidence for an independent, endogenous rhythm was obtained by Jean-Jacques d'Ortous de Mairan in as early as 1729, but it would take until the beginning of the 20th century before more experiments demonstrated the existence of a circadian clock in a diverse range of species, from invertebrates to mammals (Buijs and Kalsbeek, 2001). This clock consists of a cell autonomous transcriptional feedback loop that is present in every tissue of the body. In mammals, the suprachiasmatic nucleus in the ventral hypothalamus has the function of a central pacemaker, synchronizing all cells and tissues to the external rhythm of light and dark. It receives input from retinal ganglion cells via the retinohypothalamic tract (RHT). These cells sense light through melanopsin photoreceptors and are exclusively involved in keeping the organism entrained to the light-dark period. In a series of seminal experiments, Ralph and colleagues demonstrated the important and paramount function of the SCN in keeping physiological and behavioral rhythms synchronized. In golden hamsters, a mutation called tau affects one of the central components of the molecular clock, leading to a shortened period length under free-running (constant darkness) conditions (Ralph and Menaker, 1988). While a complete lesion of the SCN abolishes circadian rhythmicity, transplanting the SCN between

hamsters with different genotypes results in the recipient developing the rhythm of the donor (Ralph et al., 1990). The outcome is the same if the transplanted SCN is prevented, via encapsulation in a semiporous membrane, from forming new synaptic connections. These results led to the conclusion that the entrainment of basic biological rhythms is achieved through the release of diffuse neurochemical signals. Several molecules that constitute the circadian output of the SCN have been identified thus far, including TGFα, prokineticin-2 (PK2) and cardiotrophin-like cytokine (CLC) (Cheng et al., 2002; Kramer et al., 2001; Kraves and Weitz, 2006). Nevertheless, synaptic connections are still a requirement for the localized and regulated release of these signals to facilitate a fine-tuned coordination of physiology and behavior. For example, the SCN projects to nearby hypothalamic and brainstem regions, which regulate physiology in part through the rhythmic secretion of melanin and corticosteroids (Li et al., 2012a). Using a restricted feeding schedule, with food being only available for a short while during the resting period, clocks in peripheral organs can be desynchronized from the SCN, showing that other signals like feeding rhythms can entrain peripheral clocks. Other timing cues or 'Zeitgeber' include body temperature and locomotor activity.

2.3.1 The mammalian clock

The core molecular clock mechanism consists of the basic helix–loop–helix PER-ARNT-SIM (bHLH-PAS) transcription factors CLOCK and BMAL1, which bind as a heterodimeric complex to E-boxes (CACGTG/T) in the promoters of the repressors Period (Per) 1-3 and Cryptochrome (Cry) 1-2, driving their transcription. In time, PER and CRY proteins translocate back to the nucleus and inhibit their own transcription via repression of CLOCK and BMAL1. As PER and CRY proteins are continually degraded, CLOCK/BMAL1 mediated transcription can resume and the cycle starts over. The E-box controlled nuclear receptors RORα and REV-ERBα activate and suppress Bmal1 transcription, respectively, via binding to the ROR elements (ROREs) in the Bmal1 promoter (Preitner et al., 2002). The resulting rhythmic transcription of BMAL1 forms a stabilizing secondary feedback loop. By adjusting the transcriptional activity of the core clock proteins, changes in length and phase of the light-dark period

can be accommodated for. Many post-transcriptional modifications such as phosphorylation, ubiquitination and sumoylation play an important role in this process, for example by influencing protein abundance, function, and subcellular localization (Cardone et al., 2005; Eide et al., 2005; Gallego and Virshup, 2007).

Other E-box containing, clock-controlled genes (CCGs) are the PAR bZIP transcription factors albumin D-box binding protein (Dbp), thyrotroph embryonic factor (Tef) and human hepatic leukemia factor (Hlf), which constitute the main transcriptional 'output' of the core clock. (Reppert and Weaver, 2002; Schibler and Sassone-Corsi, 2002). More of a third of the mammalian genome is under circadian control (Zhang et al., 2014). All three PAR bZIP genes are cyclically expressed in the SCN. DBP and TEF accumulate in most cell types, whereas expression of HLF is restricted to liver, kidney and brain (Falvey et al., 1995; Fonjallaz et al., 1996; Reppert and Weaver, 2002).

2.3.2 *The impact of the clock on physiology and behavior*

The circadian clock regulates many, if not all, aspects of mammalian physiology. It consolidates the sleep-wake cycle by activating or deactivating the arousal systems accordingly, for example through the rhythmic expression of Dbp in orexin neurons (Akiyama et al., 2004). Another clock gene that has been linked to sleep regulation and homeostasis in particular is Period 2 (Per2). Its expression is high when EEG delta power is also high, and it increases further under conditions of sleep deprivation in proportion with time spent awake. Importantly, its expression returns to control levels after two hours of recovery sleep, confirming a likely role in sleep homeostasis. NPAS2, another core clock gene and homologue of CLOCK in the CNS, drives wake dependent expression of Per2 in the cerebral cortex, the thalamus and the cerebellum but not in the SCN.

Other functions of the circadian clock include preparing the metabolism for food uptake and increased energy expenditure and programming the immune system for increased responsiveness during the subjective day, a period of increased activity and thus increased risk of infection and injury (Curtis et al., 2014; Liu et al., 2013). Immune control by clock genes is a very complex process involving several core clock components. BMAL1 seems to be critically important in this context as a negative

regulator of the immune response. It was shown to bind to E-boxes in the promoters of chemokine ligand (Ccl) 2, Ccl8 and S100a8 (S100 calcium binding protein A8), recruiting with it members of the polycomb repressor complex (PRC2) to epigenetically mark histones for repression. Attenuation of CCL2 transcription by BMAL1 reduces numbers of infiltrating monocytes to sites of inflammation. CLOCK on the other hand has been shown to enhance the expression of TLR9, NF-κB and other genes through its intrinsic histone deacetylase activity (HAT) (Doi et al., 2006; Silver et al., 2012a; Spengler et al., 2012). Like many transcription factors, the specificity of both CLOCK and BMAL1 is dependent of the heterodimeric binding partner. In case of TLR9, CLOCK binds to E-boxes in complex with BMAL1 to epigenetically enhance the transcription of TLR9. In the case of NF-κB, CLOCK pairs with the NF-κB subunit p65 (REL A), leading to its increased phosphorylation and acetylation, and subsequently increased NF-κB activity. Another target of CLOCK is the glucocorticoid receptor, and acetylation of this receptor suppresses its binding to its target genes (Nader et al., 2009). Thus, CLOCK can upregulate immune activity through a wide range of mechanisms. The immune suppressive activity of BMAL1 may therefore be in part due to a sequestering of CLOCK (Curtis et al., 2014). Moreover, BMAL1 induces the transcription of REV-ERBα and RORα, both of which have strong proinflammatory capability (Delerive et al., 2001; Lam et al., 2013; Sato et al., 2013). PER2 on the other hand, which is anti-phasic to BMAL1, has proinflammatory function, and may in part act through the inhibition of BMAL1 and REV-ERBα (Preitner et al., 2002; Silver et al., 2012a).

Thus the clock increases the immune systems ability for detection and clearance of pathogens at the beginning of the active phase when risk of infection and injury is highest, while resolution of inflammation, tissue repair and replenishment of effector cell reservoirs is done while the organism is resting. For example, numbers of leukocytes have a strong circadian variability. In mice, they reach their highest number in the blood at Zeitgeber time (ZT) 5, which is the middle of the resting phase five hours after light onset. At ZT13, shortly after the transition to activity, leukocytes are recruited into tissues in greater numbers, which is concomitant with a higher expression of cytokines like TNF, chemotactic molecules (chemokines) and increased sensitivity to LPS (Gibbs et al., 2011; Scheiermann et al., 2012). Similarly, macrophages

show increased phagocytic activity and ability to produce cytokines during the active period (Gibbs et al., 2011; Hayashi et al., 2007).

As outlined above, the clock influences many aspects of the immune response. Conversely, the immune system can regulate the expression of many clock genes and clock-controlled genes during an inflammatory response, including clock-controlled metabolic genes (Bellet and Sassone-Corsi, 2010). For example, LPS can suppress clock gene expression and oscillation in the CNS and the liver, most likely through the induction of TNF and IL-1β (Cavadini et al., 2007; Okada et al., 2008). TNF impairs the expression of E-box driven clock genes, including the PAR bZip transcription factors Dbp, Tef and Hlf, the Per and Cry genes and REV-ERBα. These data indicate a possible crosstalk between the circadian clock and the cytokine network. Another example is the interaction of the NF-κB subunit REL-B with BMAL1 to suppress the expression of Dbp (Bellet and Sassone-Corsi, 2010). In a mouse model of collagen-induced arthritis, expression of Bmal1 mRNA was repressed in the spleen throughout the day. This most likely increased the extent of the inflammatory response, due to the potent immunosuppressant functions of BMAL1 (Hashiramoto et al., 2010).

2.3 Aim of the Project

The CD40 model of sickness behavior has only recently been established. While the role of CD40 in the immune response against pathogens and the development of autoimmune diseases has been thoroughly investigated, the molecular and cellular events underlying the physiological and behavioral changes are still poorly understood.

Since TNF plays a crucial role in CD40-mediated SBS, our goal was to find and characterize the cellular basis for this dependency. A separate but related goal was to investigate changes in sleep architecture and possible connections to cytokine-induced gene expression changes in the CNS.

3. RESULTS

3.1 Neutralization of colony-stimulating factor 1 receptor prevents sickness behavior syndrome by reprogramming inflammatory monocytes to produce IL-10

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ABSTRACT

Sickness behavior syndrome (SBS) as characterized by fatigue and depression impairs quality of life in patients with inflammatory diseases caused by infections and autoimmunity. Systemic engagement of CD40 in mice leads to an inflammatory syndrome with acute hepatitis, lymphadenopathy and development of SBS as evidenced by induction of sleep and weight loss. In the study presented here we show that the elimination of resident tissue macrophages in mice by antibody-mediated neutralization of colony-stimulating factor-1 receptor (CSF1R) did not prevent CD40 induced hepatitis, but conferred resistance to the development of SBS. The protective effect of CSF1R mAb on weight loss and behavior changes induced by CD40 activation coincided with the transformation of pro-inflammatory monocytes to IL-10 producing myeloid cells. In IL-10 knockout mice CSF1R neutralization failed to exert protection from the occurrence of SBS. This study establishes the unexpected key role of CSF1R in the polarization of inflammatory monocytes and thereby SBS in inflammatory liver diseases.

INTRODUCTION

Besides inflammation-associated organ dysfunction, patients with infections and autoimmune diseases suffer from SBS, which is characterized by fatigue, depression, weight loss and reduced social activities (Dantzer et al., 2008). CD40 ligand (CD40L) – CD40 interactions play a major role in the development of the host response to infectious pathogens and in the development of chronic inflammatory diseases including autoimmune liver diseases (Elgueta et al., 2009). CD40, a 45 to 50-kDa type I membrane glycoprotein, is expressed mainly on the surface of B lymphocytes, monocyte/macrophages, microglia and dendritic cells. CD40L (CD154) is found predominantly on activated CD4 T cells. CD40 mediates T cell dependent B cell responses, enhances the expression of MHC and co-stimulatory molecules on dendritic cells and macrophages and elicits the production of proinflammatory cytokines including tumor necrosis factor (TNF), IL-1β, IL-6, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage M-CSF/CSF-1 (Elgueta et al., 2009).

The functional relevance of CD40 expression in the initiation of inflammation was shown in mice that are treated with CD40L or with agonistic monoclonal antibodies against CD40 (CD40 mAb). Activation of CD40 induces an immune activation syndrome characterized by B cell expansion with splenomegaly, cytokine release from $CD40^+$ monocytes / macrophages and an acute, transient form of tissue inflammation, which is predominantly seen in the liver, but also affects other organs including the lung (Kimura et al., 2006; Wiley and Harmsen, 1999). In CD40 induced hepatitis, $F4/80^+/CD11b^+/Ly\text{-}6C^+$ myeloid cells, NK cells and NKT cells are recruited to the liver by CD40-activated B cells (Kimura et al., 2006). Systemic CD40 engagement also leads to SBS, which is characterized by weight loss and impairment of locomotor activity due to increased non rapid eye movement (NREM) sleep (Gast et al., 2013). Several experimental studies in rodents have demonstrated the causative role of TNF, IL-1 β and IL-6 in the induction of the these behavioral and physiological effects (Martinez et al., 2009). CD40 mAb induced production of pro-inflammatory cytokines may therefore be fundamental for the initiation of SBS.

As key producers of cytokines, monocytes and tissue-resident macrophages are potential candidates in the initiation of SBS. In response to pathogenic challenge and

signals derived from the immune system, monocytes undergo different programs of activation, rendering them either pro-inflammatory and microbicidal, or immunosuppressant and tissue repairing, the two functional types of monocytes being named M1 and M2 (Martinez et al., 2009). Of note, the strict classification of M1/M2 macrophages analogous to Th1/2 T cells does not hold up to scrutiny and has been recently revised to accommodate the dynamics and plasticity of myeloid cell function (Murray et al., 2014). During steady state conditions, resident tissue macrophages are monocytic and perform housekeeping and immune surveillance functions. In regards to their origin, there is increasing evidence that macrophages are replenished by local proliferation, with only little contribution of circulating monocytes (Hashimoto et al., 2013). This differs from the inflammatory state, which is characterized by recruitment of bone marrow derived Ly6C^{high} monocytes, followed by their differentiation into pro-inflammatory macrophages *in situ* (Ley et al., 2011). How macrophages and interacting myeloid cells are shifted from a pro-inflammatory to a resolution type of macrophage is still a matter of debate.

To assess the contribution of tissue macrophages in the pathogenesis of inflammation, monoclonal antibodies to the colony-stimulating factor-1 receptor (CSF1R mAb) have been developed. The CSF1R mAb M279 blocks the development of CD115^{+}Ly6G^{neg} monocyte precursors and inhibits the maturation, activation, survival and proliferation of differentiated resident monocyte/macrophage lineages, leading to a strong depletion of tissue macrophages (MacDonald et al., 2010). In the study presented here we find CSF1R to lead to a conversion of inflammatory monocytes to a mixed inflammatory / resolution type of cell. While liver inflammation with liver cell necrosis was unperturbed, the induction of IL-10 by CSF1R mAb was found to protect mice from CD40-driven impairment of locomotor activity and weight loss.

MATERIALS AND METHODS

Animals

Experiments were done using 10 to 12 week old male C57BL/6 IL-10$^{-/-}$ mice from Charles River, Germany. RAG1$^{-/-}$ mice were obtained from Jackson Laboratories

(USA). IL-10$^{-/-}$ mice were from Gerhard Rogler, University Zurich, Switzerland. Mice were kept at 24°C. All animal experiments were performed in accordance with relevant guidelines and regulations, and approved by the Swiss Veterinary Office, Zürich, Switzerland.

Induction of immune mediated hepatitis and SBS

Mice were treated intraperitoneally (i.p.) with 200 µl PBS containing either a monoclonal antibody to CD40 (CD40 mAb) (200 µg) or IgG2a control antibody (200 µg). Both, the CD40 mAb and the rat IgG2a were from Bio X Cell, West Lebanon, USA. The CD40 mAb is a rat monoclonal IgG2a (clone FGK45), which binds mouse CD40 and activates this receptor molecule (Rolink et al., 1996). The dose of the CD40 mAb used is the same as used in our previous studies on CD40 dependent SBS (Taraborrelli et al., 2011).

Treatment of mice with monoclonal antibodies to CSF1R

To deplete CSF1R^{+} monocytes and tissue macrophages the monoclonal rat IgG1 antibody M279 from Amgen Inc. (Thousand Oaks, CA 91320 US) was used. Mice were injected intraperitoneally 3x weekly for 3 weeks with 400 mg of either CSF1R mAb or control rat IgG1. The last injection was 1 day before CD40 mAb treatment. Control IgG1 antibodies were ordered from Bio X Cell, West Lebanon, USA.

Recording of locomotor activity and body weight

Locomotor activity was assessed by use of passive infrared sensors and freely accessible running wheels (Cavadini et al., 2007). Recordings were based on 1 min episodes using the Chronobiology Kit software (Stanford Software Systems, Santa Cruz, CA, USA). Per-minute values were averaged for each hour of recording. We allowed mice 10–15 days of adaptation to the 12:12h light–dark (L/D) cycle (lights on at 08:00 = Zeitgeber Time 0 (ZT0); lights off at 20:00 = ZT12). The locomotor activities from the three days (day -1, day -2, day -3) of recording before injection were averaged and used as baseline activity (BL). To adjust for individual differences in overall activity, raw activity scores were calibrated as percent of the mean 24 h baseline activity for each individual animal. At day 0, mice were injected i.p. at ZT5 with CD40

mAb or IgG2a control antibody. At day 2, mice were weighed killed at ZT9–10, and blood and tissues were sampled. Animals were weighed on a precision balance (Mettler-Toledo, Switzerland) before CD40 mAb or control injections and when ending the experiment at day 2.

Quantification of myeloid cells by flow cytometry

Lymphocytes and myeloid cells were isolated from mouse liver using density gradient separation as described previously (Ginhoux et al., 2009). For flow cytometry analysis (FACS), samples from bone marrow, spleen and liver were prepared and stained with CD45-APC-Cy7, CD11b-PE-Cy7, Ly6C-FITC, Ly6G-Pacific Blue, IL-10-APC and Live/Dead Aqua (Life Technologies, USA).

Analysis of liver enzymes and serum cytokines levels

Blood was harvested by cardiac puncture, allowed to clot at 4°C and serum was collected by centrifugation. Liver enzymes were measured using a Piccolo reader with Liver Panel Plus assay discs (Sysmex Digitana AG, Switzerland). Cytokine levels were measured using a magnetic bead-based ProcartaPlex assay (eBioscience, USA) on a Luminex 200 analyzer (Millipore, USA).

Statistics

For locomotor activity experiments, a two-way analysis of variance (ANOVA) with the independent variables treatment and time of day was used to test for any statistically significant difference between experimental groups. Interactions were decomposed using post hoc t-tests for group comparisons with Bonferroni correction applied for multiple comparisons. All other experiments were tested either through student`s t-test for two group comparisons or a one-way ANOVA followed by a Tukey post-hoc test for multiple group comparisons.

RESULTS

Anti-CSF1R antibodies protect from development of sickness behavior, but not from hepatitis

In the following we have assessed the extent of CD40 mediated SBS by measuring locomotor activity and body weight. Reduced locomotor activity in CD40 mAb treated mice has been shown to reflect increased sleep need with enhancement of NREM sleep (Gast et al., 2013). For simplicity, from here on, we will call impaired locomotor activity and loss of body weight "SBS". This term neglects other features of SBS, which have not yet been analyzed in CD40 mAb treated mice such as depression-like behavior.

To assess the contribution of tissue macrophages to the development of SBS, mice were injected intraperitoneally 3x weekly for 3 weeks with CSF1R mAb. Thereafter CD40 mAb were administrated. When receiving control IgG1 injections, mice displayed the typical daily variations of locomotor activity, which is highest at dark onset and decreases in the second part of the dark period (**Figure 1A**). Prolonged IgG1 injections did not prevent CD40 mAb induced severe weight loss and behavior changes as characterized by the failure to keep wakefulness during the dark period (**Figure 1A and B**). Conversely, administration of CSF1R mAb was found to protect mice almost completely from CD40 mAb-induced activity loss in the first 6 hr at ZT12-ZT18 of the dark period when normal mice are most active (**Figure 1A**). The effect was similar when recording locomotor activity by running wheel (**Figure 1A**) or by infrared sensors (data not shown). The beneficial effect of CSF1R mAb on SBS became also evident when assessing the body weight of the mice. The mean CD40 mAb induced weight loss in the control group was 12.80 ± 1.16 SEM compared to only 5.35 ± 0.91 in CSF1R mAb treated mice ($p < 0.001$) (**Figure 1B**). Since prolonged treatment of mice with the CSF1R mAb allows the depletion of $CSF1R^+$ resident tissue macrophages in e.g. liver, intestinal tract, pancreas, kidney and in the lung bronchoalveolar space without ablating the development of inflammatory monocytes (MacDonald et al., 2010), it is expected that neutralization of CSF1R would not prevent the development of CD40 mAb-mediated hepatitis. Indeed, the CD40 mAb-induced splenomegaly and increased serum ALT levels were also evident in CSF1R mAb-treated animals (**Figure 1, C and D**).

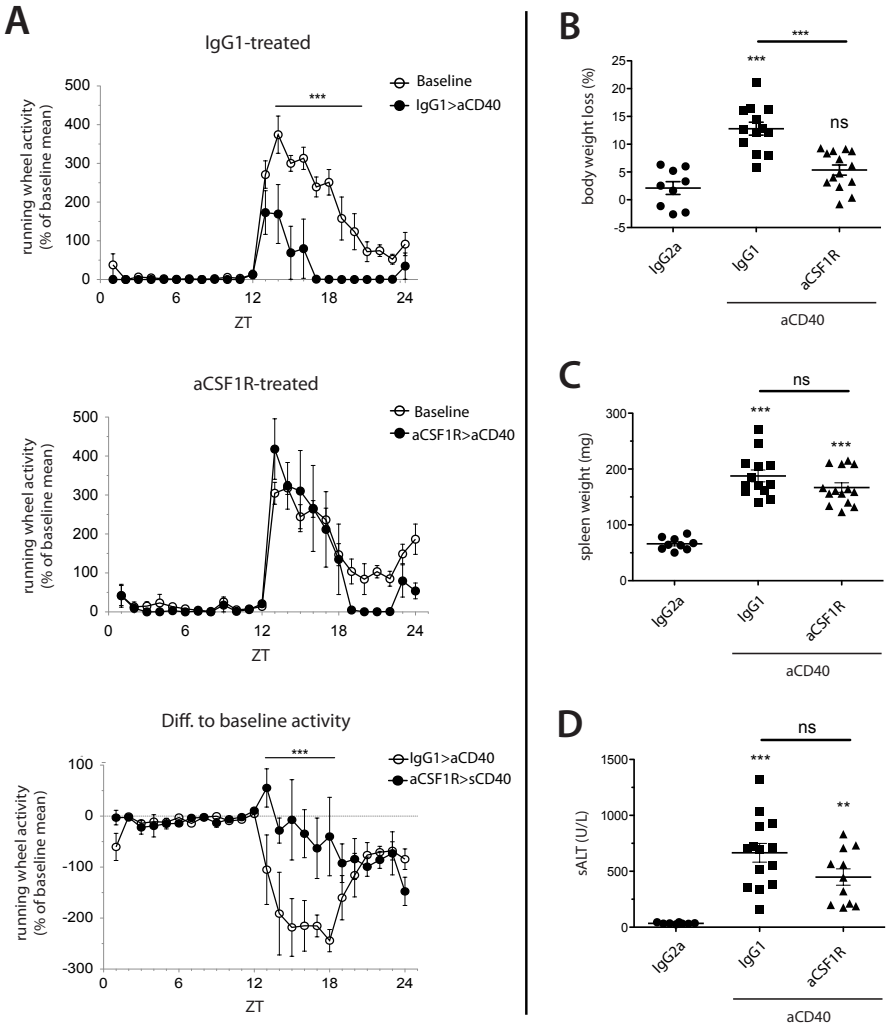

Figure 1. *CSF1R mAb treatment does not prevent hepatitis and splenomegaly, but protects from sickness behavior*

(A) Activity plots of IgG1 isotype control and CSF1R mAb (aCSF1R) treated wild-type mice. Empty dots show baseline locomotor activity, filled dots show activity at day 1 after CD40 mAb injection. Data are also given as difference to baseline activity in CD40 mAb (aCD40) treated mice with and without CSF1R mAb pre-treatment (day 1 minus baseline). Negative values represent activity loss, while positive values reflect a gain in activity. Data are shown as mean ± SEM (n=5). Two-way ANOVA with factors time, treatment, and their interaction; stars indicate the 1-h intervals that differed significantly between groups; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **(B and C)** Loss of body weight and increase in spleen weight two days after CD40 mAb or isotype IgG2a injection. **(D)** sALT levels following CD40 mAb-induced immune activation in CSFR1 mAb vs. isotype control antibody pre-treated mice. Data are mean ± SEM (n=11-14). One-way ANOVA; ** $p < 0.01$; *** $p < 0.001$;

Treatment of mice with anti-CSF1R antibodies induce the production of IL-10 in inflammatory monocytes in the liver and in the hypothalamus

As outlined above, CD40 mAb induces expression of pro-inflammatory cytokines in $CD40^+$ monocytes, macrophages and dendritic cells (Elgueta et al., 2009; Gast et al., 2013). In the following we concentrated on TNF, IL-18 and IFNγ, which are described to be essential in the CD40 mAb induced hepatitis (Kimura et al., 2011; 2006). Consistent with prior work we found CD40 mAb to increase serum levels of TNF, IL-18 and IFNγ **(Figure 2A**). Pretreatment with CSF1R mAb did not significantly alter TNF serum concentrations, but leads to a further increase in serum levels of IL-18 and IFNγ. Neutralization of CSF1R has been reported to be associated with a drastic increase of M-CSF (MacDonald et al., 2010), the effect being reproduced in the study presented here (**Figure 2A**). IL-6 serum concentrations increased in CD40 mAb treated mice, the effect not being modulated by CSF1R mAb. In the light of CSF1R mAb to inhibit CD40 induced SBS we assessed serum levels of IL-10, which is a resolution type of cytokine. While CD40 mAb treatment led to an increase of IL-10, pretreatment with CSF1R mAb further increased IL-10 serum concentrations (**Figure 2A**). Taken collectively, resistance to SBS in mice preconditioned with CSF1R mAb is not associated with decreased proinflammatory cytokines, but rather with an increase in M-CSF and IL-10.

Next we measured TNF and IL-10 mRNA in the liver as well as in the brain of CD40 mAb treated mice. The data show that CSF1R blockade induces the expression of IL-10 in the hypothalamus upon treatment of mice with CD40 mAb (**Figure 2B**). A small albeit insignificant trend towards increased expression of IL-10 was also seen in the liver. Confirming previous data, CD40 induces the expression of TNF mRNA not only in the liver, but also in the hypothalamus. CSF1R mAb on the other hand, enhanced the expression of TNF mRNA significantly in the liver, but not in the brain (**Figure 2B**).

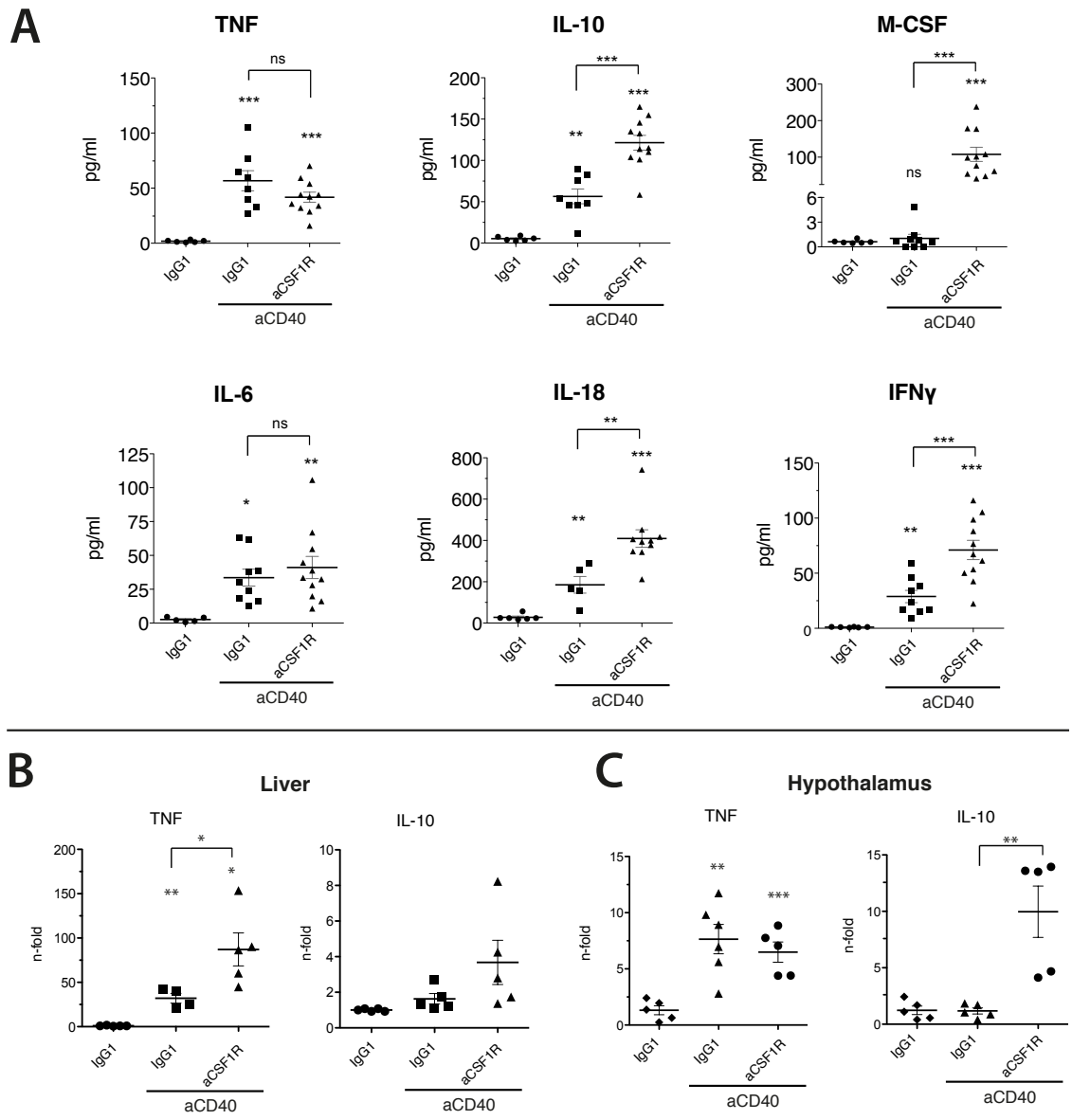

Figure 2: *Concomitant expression of pro-inflammatory cytokines, M-CSF and IL-10 in CSF1R mAb treated mice*

(A) Various pro- and anti-inflammatory cytokines were measured in serum samples of CD40 mAb and IgG2a isotype control treated mice using a magnetic bead-based Luminex assay. Data are displayed as mean ± SEM (n=5-11); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; student's t-test

(B and C) CD40 mAb-induced expression of TNF mRNA and IL-10 mRNA in the liver **(B)** and in the hypothalamus **(C)** of mice, which have been pre-treated for three weeks with CSF1R mAb (aCSF1R) or IgG1 isotype control. Thereafter mice were injected with IgG2a (Control) or CD40 mAb (CD40) and 48 hr later sacrificed and organs collected. Data are displayed as mean ± SEM (n=5); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; student`s t-test.

To evaluate the role of the crosstalk between monocytes, macrophages and neutrophils in the context of CD40 mAb-mediated hepatitis, the prevalence and immunophenotype of myeloid subsets in livers of mice pretreated with isotype control or CSF1R mAb was determined. CD40 mAb induced a massive influx of inflammatory Ly6C^{high} monocytes and neutrophils together with a loss of the F4/80^{high} macrophage population and Ly6C^{low} monocytes (**Figure 3A**). The three-week treatment with CSF1R mAb proved efficient to deplete more then 90% of liver macrophages (data not shown). This effect became also evident when mice were treated with CD40 mAb after pretreatment with CSF1R mAb. In these mice, CSF1R mAb depleted the subset of CD11b^{+}Ly6C^{neg}CD115^{+} macrophages (C), CD11b^{+}Ly6C^{+}F4/80^{high} macrophages and reduced CD11b^{+}Ly6G^{high} neutrophils (D) in the liver (**Figure 3B**). However, CSF1R mAb did not prevent CD40 mAb-induced inflammation with recruitment of CD11b^{+}Ly6C^{hi} inflammatory monocytes to the liver and liver cell injury. Furthermore, we find that CSF1R mAb-conditioning leads to an accumulation of IL-10 expressing inflammatory-like monocytes (A) and granulocytes (D) (**Figure 3, B and C**), which maintained high expression levels of TNF (data not shown), adapting a mixed pro-inflammatory / resolution profile. Indeed, a similarly mixed pro-inflammatory / resolution profile for hepatic monocytes, key players in mediating liver regeneration, has been described recently (Liaskou et al., 2013). Taken together, these data imply that tissue macrophages control the inflammatory milieu by directing the polarization of infiltrating monocytes and neutrophils.

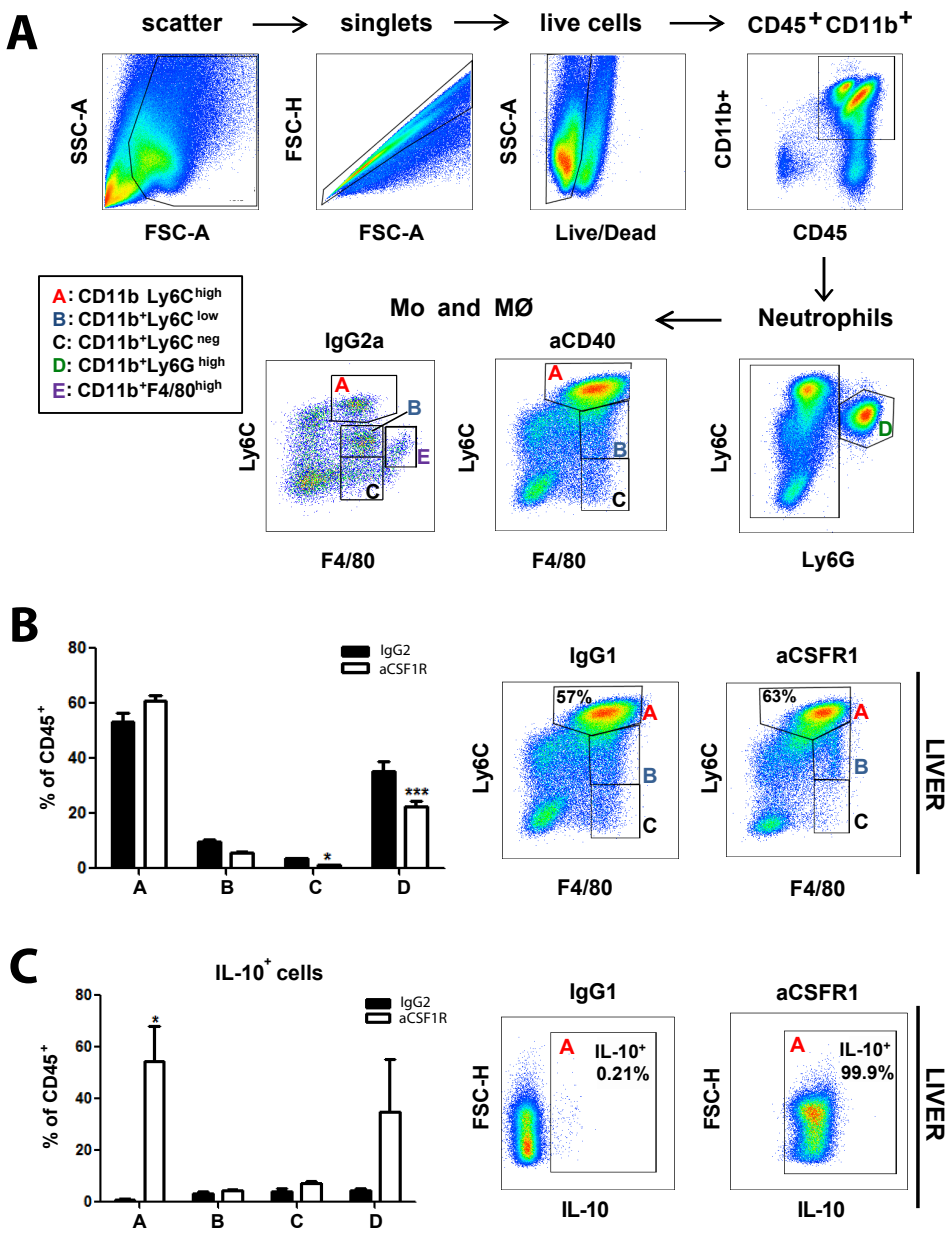

Figure 3. *Depletion of Ly6C^{neg}CD115^{+}F4/80^{+} tissue resident macrophages results in expression of IL-10 in CD11b^{+}Ly6C^{hi} inflammatory monocytes*

Mice pre-treated with CSF1R mAb or IgG1 control antibody were injected with agonistic CD40 mAb; 48 hr later livers were harvested and myeloid cells extracted and characterized. **(A)** Gating strategy for flow-based identification of myeloid subsets. Within the CD11b^{+}CD45^{+} cell gate, the CD11b^{+}Ly6G^{+}, CD11b^{+}Ly6C^{+} and CD11b^{+}F4/80^{+} cell subsets were chosen for expression analysis of intracellular IL-10. Setting gates for positive IL-10 events were defined by using FMO controls (Fluorescence Minus One). **(B)** The represented data are mean percentage of CD11b^{+}F4/80^{neg}Ly6C^{high} (A), CD11b^{+}F4/80^{neg}Ly6C^{low} (B), CD11b^{+}F4/80^{+}Ly6C^{neg} (C) and CD11b^{+}Ly6G^{hi} (D) subsets in liver from mice pre-treated with isotype IgG1 control (filled bar) or CSF1R mAb (open bar). RIGHT: Representative FACS dot plots for monocyte/macrophage subsets in liver from one mouse treated with isotype IgG1 control antibody (left) and one mouse treated with CSF1R mAb (right) are shown.

(C) The represented data are mean percentage of IL-10 expressing $CD11b^{+}F4/80^{neg}Ly6C^{high}$ (A), $CD11b^{+}F4/80^{neg}Ly6C^{low}$ (B), $CD11b^{+}F4/80^{+}Ly6C^{neg}$ (C) and $CD11b^{+}Ly6G^{hi}$ (D) of $CD45^{+}$ cells from mice pre-treated with IgG1 isotype control (filled bar) or CSF1R mAb (open bar). Right: Representative flow dot plots for $IL\text{-}10^{+}$ events in $CD11b^{+}Ly6C^{high}$ monocytes from liver of mice treated with IgG1 isotype control antibody or treated with CSF1R mAb. All data are mean ± SD; n=6; * $p < 0.05$, *** $p < 0.001$; One-way ANOVA.

IL-10 mediates protection from sickness behavior in CD40 activated mice

CD40 mAb induced SBS has been associated with increased production of TNF not only in the liver, but also in the brain where it may mediate behavioral effects (Gast et al., 2013). With IL-10 counteracting TNF production and functions, and in the light of the well-established role of IL-10 in the regulation and suppression of inflammation, we correlated the extent of rescue from CD40 mAb induced SBS by CSF1R mAb treatment with IL-10 serum concentrations in individual mice. The data show a significant negative correlation with higher IL-10 serum levels being associated with less reduction of locomotor activity (**Figure 4A**). This contrasts TNF serum levels, which were elevated independently from the extent of reduction of locomotor activity. To determine whether the mechanism of SBS suppression causatively involves IL-10, we next treated $IL10^{-/-}$ mice with CSF1R mAb. Upon induction with CD40 mAb, $IL\text{-}10^{-/-}$ mice developed hepatitis and SBS (data not shown). Likewise high numbers of granulocytes and inflammatory monocytes were found to accumulate in the liver of both $IL\text{-}10^{-/-}$ and WT mice treated with CD40 mAb (data not shown). However, CSF1R mAb failed to protect $IL\text{-}10^{-/-}$ mice from impairment of locomotor activity and weight loss (**Figure 4, B** and **C**). The effect of CD40 mAb on the relative numbers of neutrophils and inflammatory monocytes in the liver was similar in WT and $IL\text{-}10^{-/-}$ mice pre-treated with CSF1R mAb (**Figure 4D**). This indicates that the loss of protection from SBS is not due to an increased cell proliferation or infiltration, but is likely based on the inability of monocytes to produce IL-10 and adopt a resolution-like phenotype.

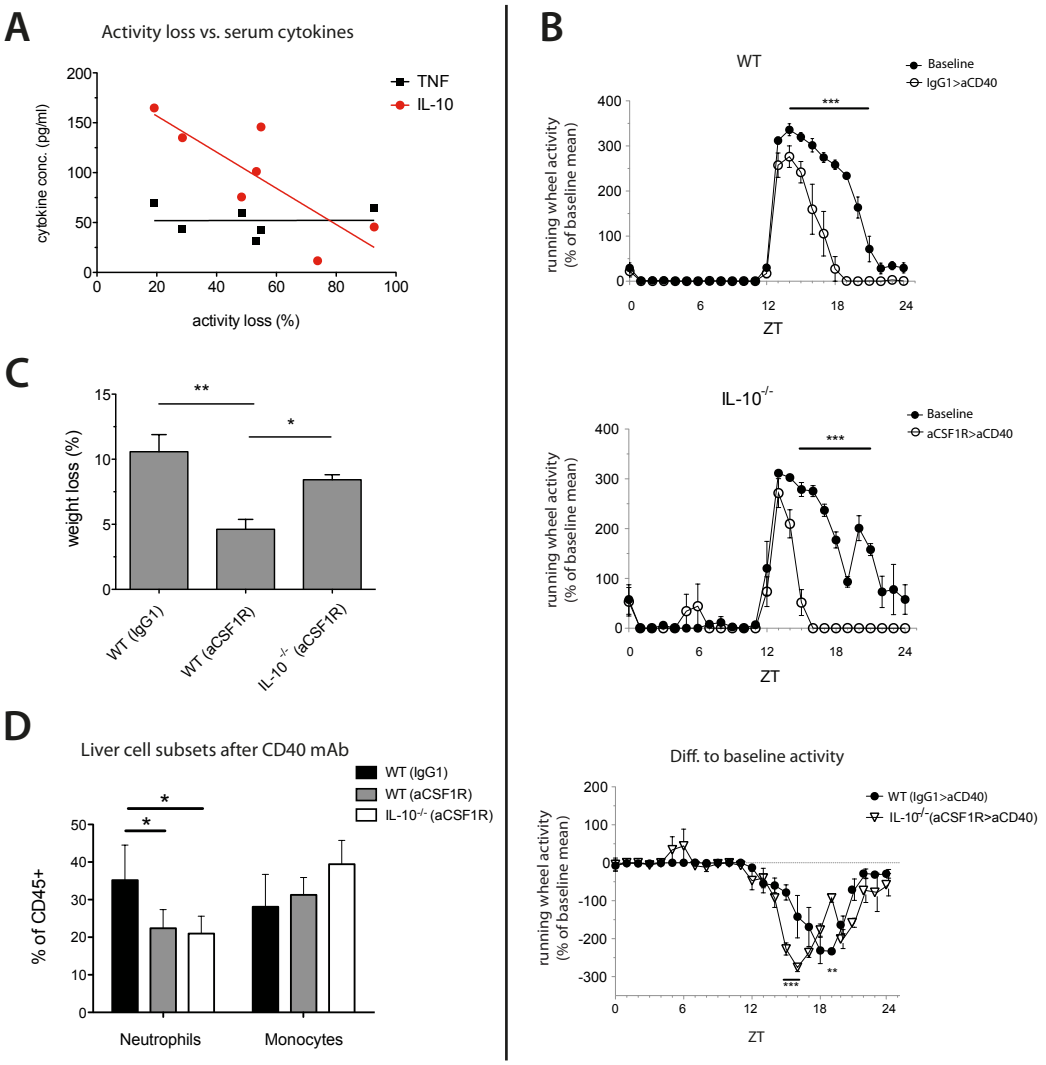

Figure 4. *CSF1R mAb fail to protect from SBS in IL-10$^{-/-}$ mice*

(A) Activity loss (%) was correlated to serum cytokine concentrations in individual CD40 mAb-injected WT mice pre-treated with CSF1R mAb (Pearson r= -0.8121 for IL-10). **(B)** Locomotor activity of WT and IL-10$^{-/-}$ mice treated with IgG1 isotype control and CSF1R mAb, respectively. Data are also given as difference to baseline activity (day 1 minus baseline). Negative values represent activity loss, while positive values reflect a gain in activity. Data are shown as mean ± SEM (n=5). Two-way ANOVA with factors time, treatment, and their interaction; stars indicate the 1-h intervals that differed significantly between groups; ** $p < 0.01$; *** $p < 0.001$. **(C)** Weight loss of WT and IL-10$^{-/-}$ mice treated with CD40 mAb. **(D)** FACS analysis of liver samples of CD40 treated mice; data are mean ± SEM, n=5; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-way ANOVA with Bonferroni correction (B) and one-way ANOVA with Tukey post-hoc test (C, D).

DISCUSSION

Neutralization of CSFR1 leads to mixed M1/M2 polarized inflammatory monocytes

Our findings reveal several interesting new aspects in the understanding of the development of SBS in inflammatory diseases. First, myeloid cells rather than T and B lymphocytes are identified here to play a pivotal role in SBS, which develops upon immune activation by CD40 signaling. Our results show for the first time that depletion of hepatic tissue resident macrophages by selective blocking of CSF1R leads to a shift of inflammatory monocytes towards a resolution-like phenotype with concomitant expression of IL-10. These monocytes might arise as a "rescue"-response triggered by the "storm" of proinflammatory cytokines observed in CSF1R mAb-treated animals and/or proinflammatory tissue macrophages are needed for conditioning incoming myeloid subsets to acquire a proinflammatory activation state. The induction of a resolution-type of monocytes by blocking CSF1R is essential in the prevention of SBS. Our data strongly suggest that CSF1R functions as key component in myeloid "re-education" in inflammation. Supporting this novel concept, it has recently been shown that blocking of M2-like tumor-associated macrophages (TAM) in glioma leads to inhibition of tumor progression by re-programming the activation status of TAMs (Pyonteck et al., 2013).

Elimination of tissue macrophages is associated with induction of IL-10 and protection from sickness behavior syndrome

Neutralization of CSF1R does not protect mice from the CD40-induced inflammatory syndrome with hepatitis, liver cell injury, lymphadenopathy, splenomegaly and increased production of proinflammatory cytokines including TNF and IL-18. These data are in agreement with the finding that blockade of CSF1R does not interfere with the recruitment of monocytes in response to intraperitoneal injection of thioglycollate broth or intratracheal instillation of lipopolysaccharide (LPS) (MacDonald et al., 2010). However, despite of systemic inflammation, CSF1R blockade protects mice from SBS as evidenced by near normal locomotor activities in the dark period of the 12 hr day / night rhythm and significantly less weight loss in affected mice. Resistance to SBS is mediated by the induction of IL-10 expression in inflammatory monocytes. This conclusion is based on the observation that CSF1R mAb failed to prevent the

development of SBS in IL-10$^{-/-}$ mice. IL-10 may counteract the development of SBS by interfering with the action of proinflammatory cytokines. TNF, IL-1β and IL-18 have been well described to induce SBS, including weight loss and NREM sleep, when injected systemically into rodents (for review see Dantzer et al., 2008).

To understand the effect of CSF1R blockade to prevent SBS by induction of IL-10 in inflammatory monocytes, it is important to note that, while depleting tissue macrophages in most organs, CSF1R mAb do not deplete microglia in the brain parenchyma (MacDonald et al., 2010). The expression of CD40 by microglia has been implicated in the cytokine-induced enhancement of the immune response in the central nervous system (CNS) (Ponomarev et al., 2006). Antibodies against CSF1R and CD40 show only limited diffusion from the vasculature into the CNS parenchyma. However, CD40 mAb and TNF may damage the endothelial cell barrier and thereby allow trafficking of inflammatory monocytes into the brain parenchyma and activate resident microglia. Surprisingly, in recent studies we failed to detect infiltration of Mac-3^{+} monocytes or CD3^{+} T and B220^{+} B lymphocytes into the brain of CD40 mAb injected mice (Gast et al., 2013). Moreover Mac-3 and Iba-1 staining did not reveal signs of microglia activation. This does not exclude that CD40 mAb lead to an increase of the permeability of the BBB whereby cytokines such as TNF may reach the brain parenchyma and, as shown previously, mediate their own transcription (Laflamme and Rivest, 1999; Qin et al., 2007). Using transmission electron microscopy and tracers to assess BBB functioning, treatment of mice with D-galactosamine and LPS was found to disrupt tight junctions, the effect being induced by TNF and associated with downregulation of the tight junction protein occludin (Lv et al., 2010). Likewise, upregulation of TNF in CD40 mAb treated mice may allow cytokines to diffuse throughout perivascular spaces, which are occupied by macrophages, and reach their target cells including microglia (Konsman et al., 2008). Analogously M-CSF/CSF-1, which as shown in the present study is highly increased in serum of CSF1R mAb treated mice, may reach microglia in the brain and trigger IL-10 production. This scenario is based on the observations that (i) the interaction of M-CSF/CSF-1 with macrophages stimulates IL-10 production (Verreck et al., 2004), (ii) as shown here, IL-10 expression is increased in the hypothalamus of mice treated with CSF1R mAb and challenged with CD40 mAb, and (iii) that, dependent on the activation signal used,

microglia cells produce IL-10 (Aloisi et al., 1999; Lambert et al., 2008). Intracerebroventricular injection of IL-10 has been shown to reduce spontaneous and LPS-induced NREM sleep and loss of social exploration in rodents (Bluthé et al., 1999; Opp et al., 1995). Compared to WT mice, IL-$10^{-/-}$ mice respond to LPS with a prolonged increase of slow wave sleep, and showed more pronounced impairment of cognitive deficits, locomotor activity and food intake (Toth and Opp, 2001). An imbalance between pro-inflammatory and anti-inflammatory activity with decreased IL-10 was also observed in patients with untreated major depressive disorder, in patients with bipolar disorders and in depression associated with colorectal cancer or stroke (references are given in Voorhees et al., 2013). The IL-10-dependent pathways involved are not known.

CONCLUSION

Sickness behavior syndrome (SBS) with fatigue and depression impairs quality of life in patients with inflammatory syndromes caused by infections or autoimmunity. What remains unresolved is whether SBS is an inevitable, unspecific consequence of tissue inflammation per se, or whether a specific molecular pathway, which is disconnected from inflammation, governs the behavioral changes. The study presented here uses a new immune regulatory circuit to ameliorate changes in behavior during inflammatory diseases. We show that the elimination of tissue macrophages with blocking CSF1R induces the expression of IL-10 in inflammatory monocytes, which arise when activating the CD40 pathway in mice. Despite of severe hepatitis with increased expression of proinflammatory cytokines, mice become protected from development of sickness behavior. The increased expression of IL-10 in CSF1R antibody treated mice overrides the negative effects of proinflammatory cytokines on behavior and body weight. These data provide a major conceptual advance in understanding the molecular and cellular events leading to SBS.

Author contributions

A.F.M., and L.S., designed and performed the experiments. M.G., H.G., M.R., B.B., and A.F. contributed to the concept of the study.

Conflict of interest

The authors have declared that no conflict of interest exists.

Acknowledgments

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SUPPLEMENTARY INFORMATION

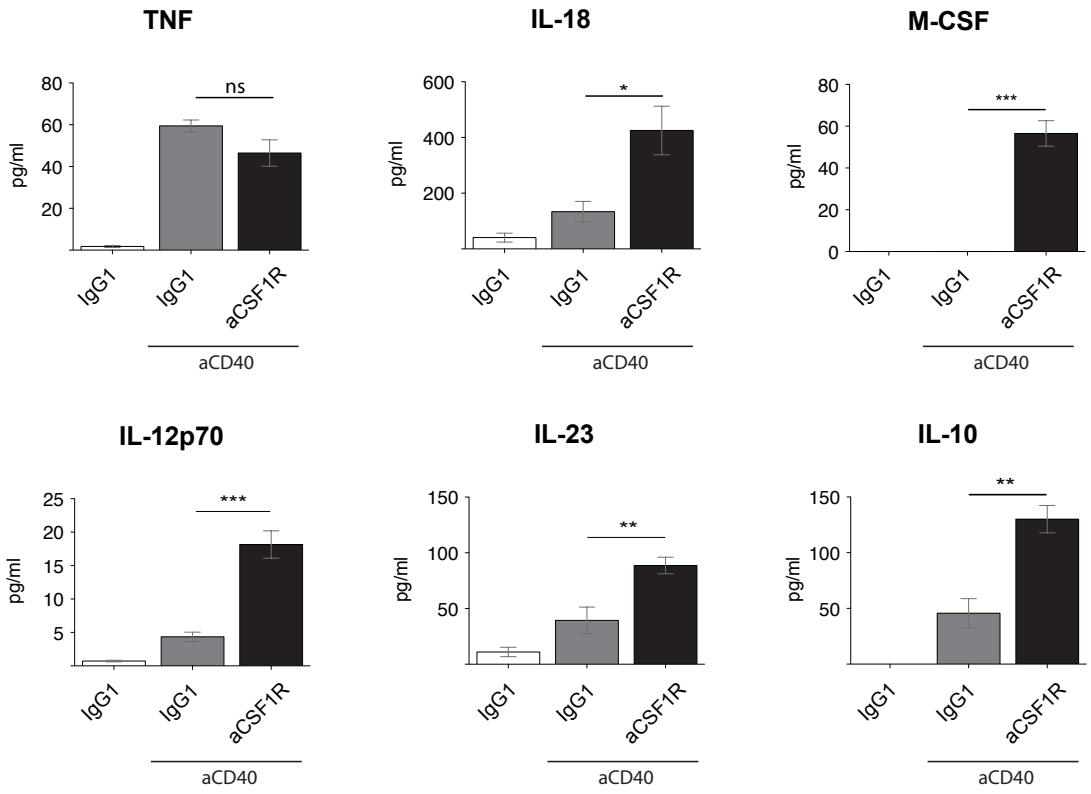

Figure S1. *CSF1R mAb pre-treatment alters the expression of cytokines.* Various pro- and proinflammatory cytokines were measured in serum samples of CD40 mAb and IgG2a isotype control treated mice using a magnetic bead-based Luminex assay. The marked increase of M-CSF has been described to reflect efficacy of the CSF1R mAb treatment (MacDonald et al., 2010). Data are displayed as mean ± SEM (n=6); * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; student's t-test.

3.2 CD40 activation induces NREM sleep and modulates genes associated with sleep homeostasis

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ABSTRACT

The T cell-derived cytokine CD40 ligand is overexpressed in patients with autoimmune diseases. Through activation of its receptor, CD40 ligand leads to a tumor necrosis factor (TNF) receptor 1 (TNFR1) dependent impairment of locomotor activity in mice. Here we report that this effect is explained through a promotion of sleep, which was specific to non-rapid eye movement (NREM) sleep while REM sleep was suppressed. The increase in NREM sleep was accompanied by a decrease in EEG delta power during NREM sleep and by a decrease in the expression of transcripts in the cerebral cortex known to be associated with homeostatic sleep drive, such as Homer1a, Early growth response 2, Neuronal pentraxin 2, and Fos-like antigen 2. The effect of CD40 activation was mimicked by peripheral TNF injection and prevented by the TNF blocker etanercept. Our study indicates that sleep–wake dysregulation in autoimmune diseases may result from CD40-induced TNF:TNFR1 mediated alterations of molecular pathways, which regulate sleep–wake behavior.

INTRODUCTION

In autoimmune diseases the destruction of target cells in various organs is caused by the action of anti-self T cells, autoantibodies and activated macrophages. Besides organ dysfunction however, patients also suffer from sickness behavior syndrome (SBS), which is characterized by fatigue, malaise, decreased appetite, weight loss and reduced social activities (Dantzer et al., 2008). In multiple sclerosis, autoimmune hepatitis, rheumatoid arthritis, systemic lupus erythematosus (SLE), and inflammatory bowel disease, patients suffer from fatigue that is a significant comorbidity severely affecting quality of life. SBS is also frequently seen in acute or chronic infections and has been assumed to be due to the action of cytokines (Krueger, 2008; Opp, 2005). When administered peripherally or directly into the cerebral ventricles, cytokines including TNF, interleukin (IL)-1β, IL-6, and interferons induce SBS. Likewise SBS, which follows activation of Toll-like receptor (TLR) 3 with synthetic double-stranded RNA (polyI:C) or stimulation of TLR4 with lipopolysaccharide (LPS), is associated with an increase in cytokine production both in the central nervous system (CNS) and in the immune compartment (Bluthé et al., 2000; Cunningham et al., 2007). To study the mechanism of SBS in autoimmune diseases we have developed a new animal model using the CD40–CD40 ligand (CD40L) pathway of immune activation. CD40 is expressed mainly on antigen presenting cells, B-lymphocytes and endothelial cells (for review see Grewal and Flavell, 1998). CD40 mediated signaling is induced by CD40L, which is expressed by activated $CD4^+$ T-cells. CD40 activation leads to production of cytokines including TNF, IL-1β, IL-6, IL-8, interferon (IFN) α, β and γ, IL-12, IL-17, IL-23, and granulocyte-macrophage colony stimulating factor (Danese et al., 2004; Elgueta et al., 2009). CD40L:CD40 interaction is essential for the autoimmune response to self antigens. It was demonstrated that inhibition of CD40 or CD40L renders mice resistant to the induction of experimental autoimmune diseases (for review: Peters et al., 2009). On the other hand, increased signaling via CD40L:CD40 has been observed in multiple sclerosis, rheumatoid arthritis, SLE, and in HIV infected patients, diseases that are well known to be associated with fatigue (Peters et al., 2009; Sui et al., 2007). SBS is also induced in patients with chronic lymphatic leukemia or multiple myeloma when treated with monoclonal antibodies (mAb) to CD40 (Advani et al., 2009; Furman et al., 2010; Hussein et al., 2010). Our previous studies in mice show that activation of CD40

leads to SBS (Taraborrelli et al., 2011). The latter is characterized by weight loss and reduced locomotor activity. Interestingly, mice with an inactivation of the TNF receptor 1 gene ($Tnfr1^{-/-}$) are completely protected from CD40-mediated SBS, but not from immune activation (Taraborrelli et al., 2011). This study made use of a monoclonal antibody to CD40 (CD40 mAb), which by binding to this cell surface receptor molecule activates intracellular signaling pathways and thereby activates macrophages to produce cytokines (Fig. 1). Injection of the CD40 mAb into mice mimics the effects seen in transgenic mice overexpressing CD40L. Activation of CD40 by CD40L or by CD40 mAb leads to a wasting syndrome with inflammation and hypertrophy of lymphoid tissues as characterized by loss of normal follicular structure and colocalization of activated T cells and B cells in large lymphocytic clusters. Lymphadenopathy and splenomegaly is also due to myeloid hyperplasia. Moreover pronounced infiltrates of B cells, NK cells, CD4 T cells, dendritic cells and macrophages, are seen in various organs including lung, liver, pancreas and gastrointestinal tract (Clegg et al., 1997; Kimura et al., 2006). There is substantial evidence that TNF and IL-1β enhance non-rapid eye movement (NREM) sleep when injected into animals (Krueger, 2008; Opp, 2005; Shoham et al., 1987). Moreover in rats Tnf mRNA brain levels vary with sleep propensity (Bredow et al., 1997; Floyd and Krueger, 1997). Furthermore, reduction of Tnf mRNA by microinjection of Tnf short interfering RNA (siRNA) into the rat primary somatosensory cortex (SSctx) reduced EEG delta power during NREM sleep at the site of injection, but not contralaterally (Taishi et al., 2007). EEG delta power (1–4 Hz) is widely used as a state variable of the sleep homeostatic process (Borbély and Achermann, 1999). Local application of TNF onto the SSctx induces EEG asymmetry with increased activity in the delta frequencies during NREM sleep, while the duration of NREM does not change (Yoshida et al., 2004). The response to sleep deprivation (SD) has also been found to involve TNF. Pretreatment of rabbits with fragments of human recombinant soluble TNF receptor 1 and of human type I IL-1 receptor attenuated the sleep rebound after SD (Takahashi et al., 1996a; 1996b). Taken collectively, TNF may not only be involved in SBS, but is likely to also play a role in physiological sleep regulation (Krueger, 2008). At the molecular level the homeostatic regulation of sleep has been studied intensively using SD. SD leads to increased expression of, e.g., immediate early genes/transcription factors, mitochondrial genes, genes involved in

energy metabolism, and neurotransmitter transporters and receptors (Cirelli, 2009). Transcriptome profiling in inbred mouse strains show that genetic background affects susceptibility to SD at the transcriptional level. When taking the genetic background into account, expression of Homer1a associates with changes in homeostatic sleep need (Franken et al., 2001; Mackiewicz et al., 2008; Maret et al., 2007). Neurons expressing Homer1a also express early growth response 2 (Egr2/Krox20), Fos-like antigen 2 (Fosl2), prostaglandin-endoperoxide synthase 2 (Ptgs2), junctophilin 3 (Jph3), and neuronal pentraxin 2 (Nptx2) (Maret et al., 2007). Expression of these transcripts equally increases with sleep need. In this study, we analyzed the effect of CD40 mAb on sleep–wake behavior in mice. Moreover we investigated the role of TNF in the response to CD40 mAb and assessed the expression of genes described to increase in the cerebral cortex with sleep need.

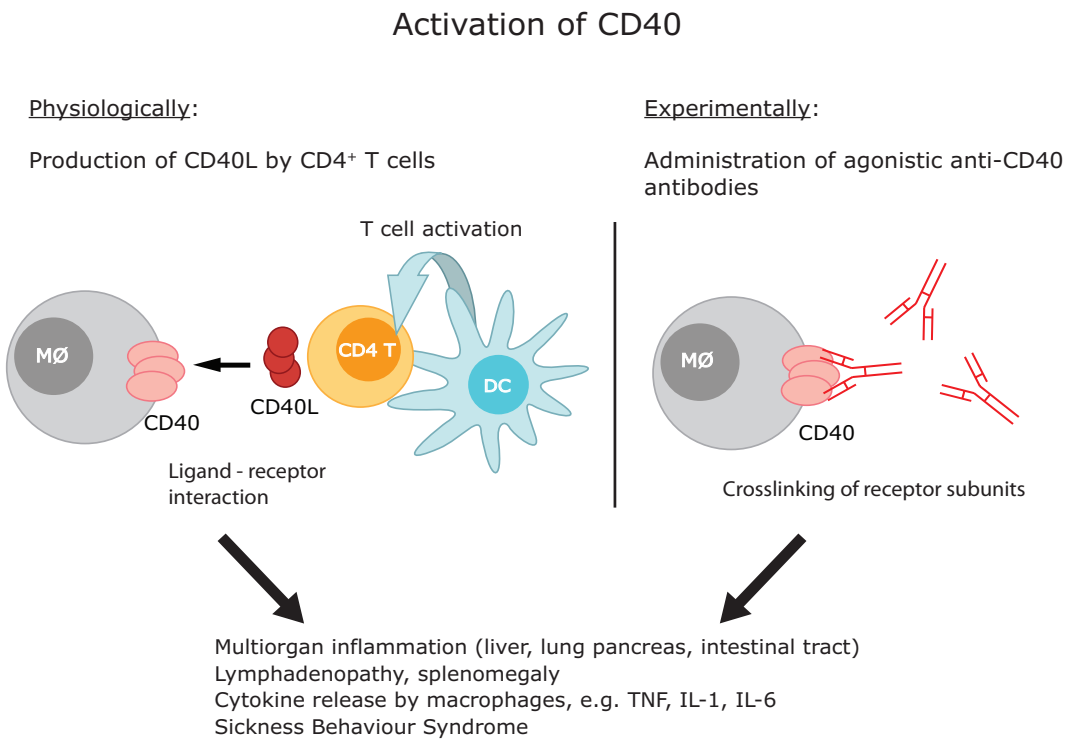

Figure 1: Activation of CD40 by CD40 ligand or agonistic anti-CD40 antibodies leads to multiorgan inflammation and sickness behavior syndrome. Antigen presentation by dendritic cells to MHC class II restricted CD4 T cells induces the production of CD40L, which bind as homotrimers to CD40 expressed on macrophages and dendritic cells. Binding of CD40L–CD40 molecules, which are also expressed in trimeric forms, activates the cells as evidenced by induction of migration, upregulation

of MHC class II and adhesion molecules as well as production of chemokines and cytokines. Among the different anti-CD40 monoclonal antibodies available, there are some that induce intracellular signaling and thereby mimic the effect of CD40L. Both CD40L and the agonistic CD40 mAb promote multiorgan inflammation and sickness behavior syndrome.

MATERIALS AND METHODS

Mice

Experiments were done using 10–12 week old male C57BL/6 mice from Charles River, Germany. Mice were kept at 24°C. All animal experiments were performed in accordance with relevant guidelines and regulations, and approved by the Swiss Veterinary Office, Zurich, Switzerland.

Treatment of mice with antibodies and etanercept

Fig. 2 shows the timing of the experiments, which assess the effects of CD40 mAb, IgG and etanercept on locomotor activity, sleep and gene expression. Mice were treated intraperitoneally (i.p.) with 200 µl PBS containing 200 µg of either a monoclonal antibody to CD40, IgG2a control antibodies or IgG2a plus etanercept (200 µg each). Both the CD40 mAb and the rat IgG2a were obtained from Bio X Cell, West Lebanon, NH, USA. The CD40 mAb is a rat monoclonal IgG2a (clone FGK45), which binds mouse CD40 and activates this receptor molecule (Rolink et al., 1996). The dose of the CD40 mAb used here is in line with the doses being applied in other in vivo studies (100–300 µg/day) (Blair et al., 2009; Serra et al., 2003). Etanercept consists of the binding part of the TNFR2 linked to the Fc portion of IgG (Peppel et al., 1991). In selected experiments, mice were injected i.p. with recombinant murine TNF (2 µg, Peprotech, UK) diluted in 200 µl PBS + 0.1% BSA or with diluent alone.

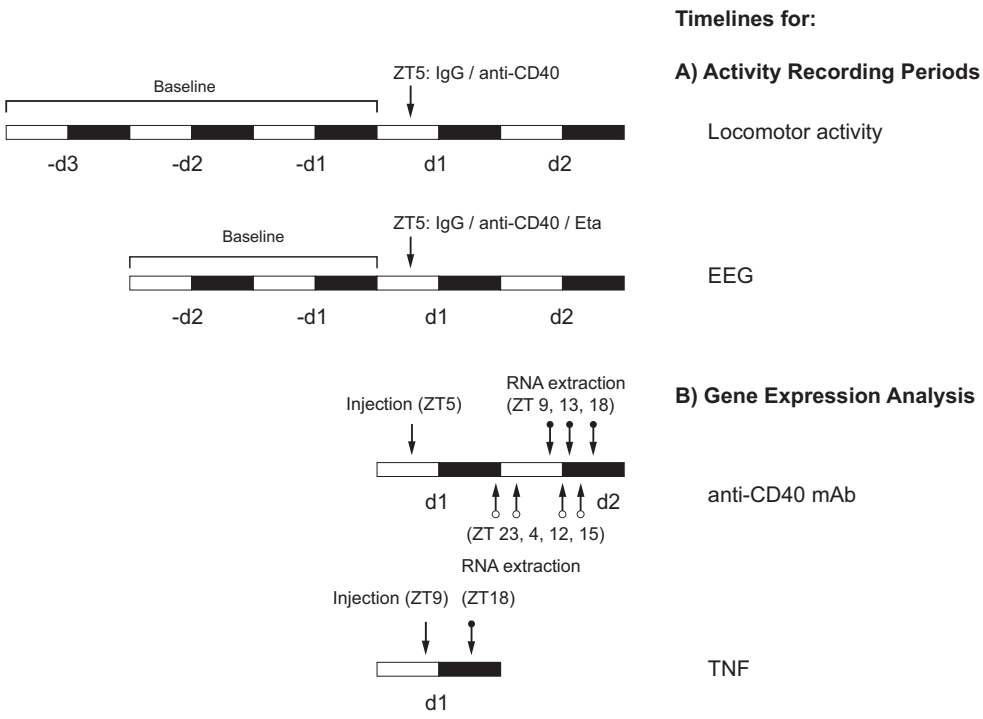

Figure 2: Timing of experiments, which assess the effects of CD40 mAb, IgG and etanercept on locomotor activity, sleep and gene expression. Gene expression following treatment of mice with CD40 mAb was done either on day 1 (d1) at Zeitgeber (ZT) 23 and d2 at ZT4, ZT12 and ZT15 (open arrows) or at d2 at ZT9, ZT13 and ZT18 (closed arrows).

Locomotor activity recording

Locomotor activity was assessed by use of passive infrared sensors and freely accessible running wheels (Cavadini et al., 2007). Recordings were based on 1 min episodes using the Chronobiology Kit software (Stanford Software Systems, Santa Cruz, CA, USA). Rest episodes were defined as 1 min units with zero activity. We allowed mice 10–15 days of adaptation to the 12:12 h light–dark (L/D) cycle (lights on at 08:00 = Zeitgeber Time 0 (ZT0); lights off at 20:00 = ZT12). The locomotor activities from the three days (-day 1, -day 2, -day 3) of recording before injection were averaged and used as baseline activity (BL). At day 1, mice were injected i.p. at ZT5 with antibodies and etanercept or IgG2a control antibody. At day 3, mice were weighed and killed at ZT9-10 and tissues were taken. In selected experiments TNF or diluent control was injected at ZT9 of the light period and brains taken for RNA extraction 9 h later at ZT18.

EEG/EMG electrode implantation

Mice were implanted epidurally with gold-plated screws (0.9 mm diameter) for EEG recording under isoflurane anesthesia (right hemisphere: above the frontal cortex, 1.5 mm anterior to bregma, 2 mm lateral to midline, and the parietal cortex, 2 mm posterior to bregma, 3 mm lateral to the midline; reference: above the cerebellum, 2 mm posterior to lambda, on the midline). Two gold wires (0.2 mm diameter) were inserted bilaterally next to the neck muscles for EMG recordings. The electrodes were connected to stainless steel wires fixed to the skull with dental acrylic cement. To provide a maximum degree of freedom of movement, mice were connected to a swivel by a fine cable. At least 2 weeks were allowed for recovery from surgery before starting the experiments (Palchykova et al., 2010).

Experimental protocol in EEG studies

Mice were kept in soundproof and light-tight recording chambers with a 12:12 h light–dark cycle (ZT0 = lights on) for the duration of the experiment. EEG/EMG activity was recorded over a period of 4 consecutive days, with the first 48 h serving as baseline reference. At day 1, mice were injected i.p. at ZT5 with CD40 mAb, IgG2a control antibodies and etanercept.

EEG data acquisition and analysis epochs

EEG signals were recorded and analyzed as previously described (Hasan et al., 2009) using commercial hard- (EMBLA™) and software (Somnologica-3™; Medcare/Flaga, Island). The analogous EEG and EMG signals were digitized at 2000 Hz and subsequently stored at 200 Hz on hard disc. The EEG was subjected to a discrete Fourier transformation, yielding power spectra (range: 0.25–90 Hz, resolution: 0.25 Hz, window function: hamming) for consecutive 4 s epochs. EEG delta power (1–4 Hz) during NREM sleep was calculated for percentiles of time (12 and 6 for the light and dark periods, respectively) to which an equal number of NREM sleep episodes contributed and then expressed as % of the individual levels reached in the last 4 h of the light periods. The EMG was fullwave rectified and integrated over 4 s epochs. Sleep fragmentation was quantified by counting the number of brief awakenings (nBA < 16 s), the number of NREM sleep episodes shorter than 60 s, and the number of NREM

sleep episodes longer than 60 s according to Franken et al., 1999. These numbers were corrected for differences in sleep time by expressing them per h of NREM sleep. Quantitative data analysis was performed using the MATLAB™ software package (The MathWorks, Natick, Mass., USA) and SAS (SAS Institute Inc, Cary, NC, USA). For the data that is shown in Fig. 3 the three vigilance states (NREM sleep, REM sleep, and waking) were determined off-line by visual inspection of the parietal and frontal EEG, and EMG records for 4s epochs.

Statistical analysis

To determine the effects of CD40 mAb on sleep two-way analysis of variance (ANOVA) with factors treatment and time was performed. Significant interactions were decomposed using post hoc t-tests for two group comparisons and Tukey's HSD tests for multiple group comparisons for sleep fragmentation variables. Statistical analyses were done with SAS (SAS Institute Inc, Cary, NC, USA) and MATLAB™ (MathWorks, Natick, Mass, USA). Statistical significance was set to $p = 0.05$ and results are reported as mean ± SEM. The effects of etanercept on CD40 mAb induced sleep were determined performing an ANOVA to examine the main effects and interactions of the three experimental factors: group (control, CD40, and CD40/Eta); night-half (first and second half of the night); and measurement-day (day 1 or day 2). Post-hoc effects were individually tested using multiple independent Mann–Whitney U-tests. These statistical analyses were done with SPSS (SPSS Inc. Chicago IL). Likewise for comparisons of mRNA levels, TNF serum concentrations, changes in body weight and spleen weight, multiple independent Mann–Whitney U tests were used.

RNA isolation and gene expression analysis

Gene expression analysis was performed according to Petrzilka et al. (2009). RNA from mouse tissues was extracted by homogenization of the organ in peqGOLD RNA pure (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. Isolated RNA was thereafter DNA digested and purified with spin columns NucleoSpin RNA II (Macherey-Nagel, Düren, Germany). Then cDNA was synthesized using random hexamers (Applied Biosystems, AB, Rotkreuz, Switzerland) and M-MuLV reverse transcriptase (AB). The cDNA equivalent to 20 ng of total RNA was PCR-amplified in

an ABI PRISM HT7900 detection system (AB) using the TaqMan Universal PCR Master Mix (AB) and quantified as previously described (Petrzilka et al., 2009). All samples were run in duplicate and results were normalized to 18S rRNA (AB). Relative mRNA levels are expressed as n-fold variations compared with the control treated group or as percent expression with the highest expressor set to 100%. The time course of expression of the genes analyzed are provided as the $2^{-\Delta CT}$ values (where ΔCT = CT gene of

interest - CT internal control) in means ± SEM. TaqMan assays were from AB; 18s rRNA, 4310893E; Ptgs2, Mm01307330_g1; Jph3, Mm00517489_m1; Nptx2, Mm00479438_m1; Egr2, Mm00456650_m1; Fosl2, Mm00484442_m1; Tnf, Mm00443258_m1; Il-1β, Mm00434228_m1; Homer1a as described (Maret et al., 2007).

Measurement of TNF in brain tissue

For total protein extraction and TNF measurements we used a modified protocol adapted from methods previously described for analysis of cytokines in the brain (Datta and Opp, 2008; Erickson and Banks, 2011). Mice were sacrificed; perfused transcardially with ice-cold PBS and the left and right frontal cortices and cerebella were dissected and immediately snap-frozen in liquid nitrogen. The brain samples were homogenized with QIAGEN Tissue Lyser II for 2.5 min/25 Hz in extraction buffer (20mMTrisHCl, 0.15 M NaCl, 2 mM EDTA, 1 mM EGTA, and a protease inhibitor cocktail (Sigma, St. Louis, MO)). Samples were centrifuged (1000 x g for 10 min at 4°C.); thereafter the supernatants were removed and centrifuged a second time (16,000 x g for 120 min at 4°C.). Protein levels were quantified with the Bradford protein assay (BIO-RAD). TNF measurements were performed by a Luminex-based system using Bio-Plex Pro™ assay (BIO-RAD).

Tissue preparation and immunohistochemistry

Forty-eight hours after i.p. injection of CD40 mAb mice were sacrificed with CO_2 and histology performed as described recently (van Loo et al., 2006). The brains were removed and fixed in 4% buffered formalin. Thereafter brains were dissected and embedded in paraffin before staining with antibodies to MAC-3 (BD Pharmingen) and Iba-1 (WACO, Japan) for macrophages/microglia, CD3 for T lymphocytes (Serotec,

Düsseldorf, Germany) and B220 for B lymphocytes (BD Pharmingen). Brain tissues were then incubated with secondary antibody biotin conjugated polyclonal rabbit anti-rat antibodies (Dako) followed by streptavidin biotin-horseradish peroxidase complex (Calbiochem) (Dann et al., 2011).

RESULTS

Etanercept protects mice from CD40 mediated impairment of locomotor activity

Since TNFR1 gene knockout mice have been described to be resistant to CD40-mediated SBS (Taraborrelli et al., 2011), we explored whether blockade of TNF-mediated signaling by etanercept would interfere with the behavioral effect of CD40 activation. Confirming recent observations, we found that mice injected i.p. with CD40 mAb showed a profound reduction of locomotor activity at dark and a significant loss of body weight when compared to IgG2a and etanercept treated mice or to untreated mice (Fig. 3B and E) (Taraborrelli et al., 2011). TNF blockade with etanercept profoundly suppressed both effects of CD40 mAb, the impairment of locomotor activity, and the reduction of weight (Fig. 3C–E). Resistance to the effect of CD40 mAb is not due to interference of etanercept with immune activation because, despite etanercept treatment, CD40 mAb still provoked a 4-fold increase in spleen weight (Fig. 3F).

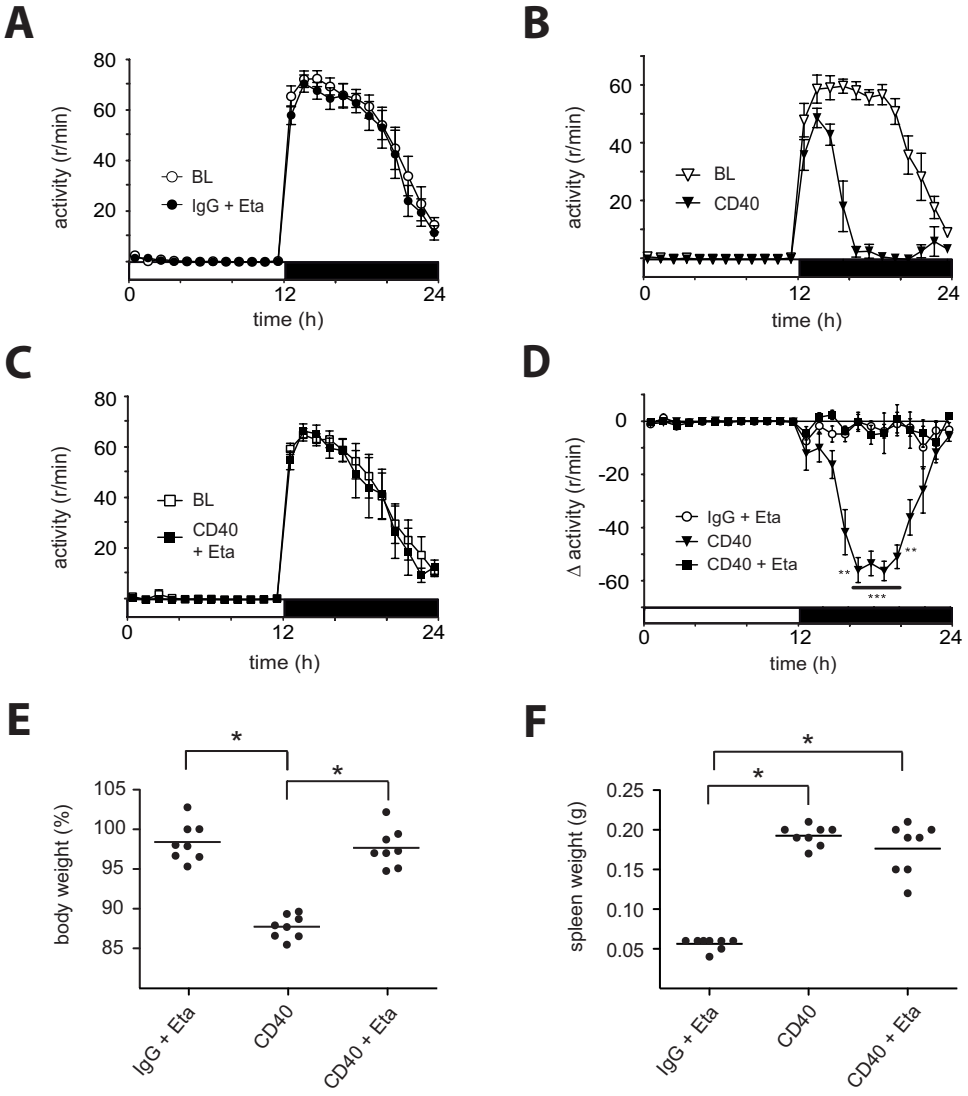

Figure 3: Etanercept prevents CD40 mAb induced loss of locomotor activity and body weight. Running wheel activity recorded at day 2 after i.p. injection of C57Bl/6 mice with (A) IgG2a control antibody plus etanercept (Eta) (closed circles), or (B) with CD40 mAb (closed triangles) or (C) CD40 mAb plus Eta (closed squares). Baseline (BL data: open symbols (A, B and (C) are the mean of 24 h recordings of three consecutive days prior to injection of antibodies in each group. Data show the means of 1 h values ± SEM. (D) Difference in running wheel activity after subtracting the baseline values; open circles: IgG2a plus Eta; closed triangles: CD40 mAb; closed squares: CD40 mAb plus Eta. The data is shown as means ± SEM. Stars indicate the 1-h interval which differed significantly between CD40 mAb treated and BL groups: two-way ANOVA with factors ''interval'' and ''treatment''; $F = 17.93$. ** $p < 0.005$, *** $p < 0.0005$, post hoc two-tailed t-test (E) Etanercept prevents CD40 mAb induced weight loss. Change in body weight compared to pre-injection is shown in percent of each individual (n = 8/group; Mann–Whitney U test). (F) Etanercept has no effects on splenomegaly induction by CD40 mAb treatment. Data show means ± SEM. Number of mice: 8 per group. * $p < 0.05$.

Activation of CD40 alters sleep structure, an effect that can be prevented by etanercept

Analysis of sleep–wake behavior shows that CD40 mAb treatment suppressed locomotor activity through a promotion of sleep. The increase in sleep was specific to NREM sleep while REM sleep was suppressed. The effect of CD40 activation on locomotor activity and NREM sleep became evident in the second half of the first dark period; i.e. 13 h after injection, and was most pronounced in the second dark period (Fig. 4A). The CD40 mAb mediated suppression of REM sleep was at maximum in the light period of day 2 after CD40 mAb treatment. Analyses of the accumulation of the hourly differences of NREM sleep and REM sleep levels versus individual corresponding baseline values revealed that 7.2 h extra NREM sleep was accrued over the 43 h time period post CD40-injection compared to controls (Fig. 4B). During the light period of the second day after CD40 mAb treatment, mice lost 36min of REM sleep of which 10 min were recuperated during the subsequent dark period. The opposing effects on NREM sleep and REM sleep were summarized by expressing REM sleep as a percentage of total sleep time (NREM and REM sleep). During the light period of day 2, the contribution of REMS to sleep was reduced from 14.2 ± 0.4% in controls to 4.3 ± 0.7% in CD40 mAb treated mice; i.e., a 3.3-fold reduction (post hoc t test, $p < 0.0001$, $n = 5$/treatment). Analysis of the time course of EEG delta power during NREM sleep showed delta power to be low and without variation with time on the second day after CD40 mAb administration (Fig. 4C). This decrease was accompanied by an increase in sleep fragmentation with greatly increased numbers of brief awakenings (nBA) and of short NREM sleep episodes and less longer NREM sleep episodes (Fig. 4D).

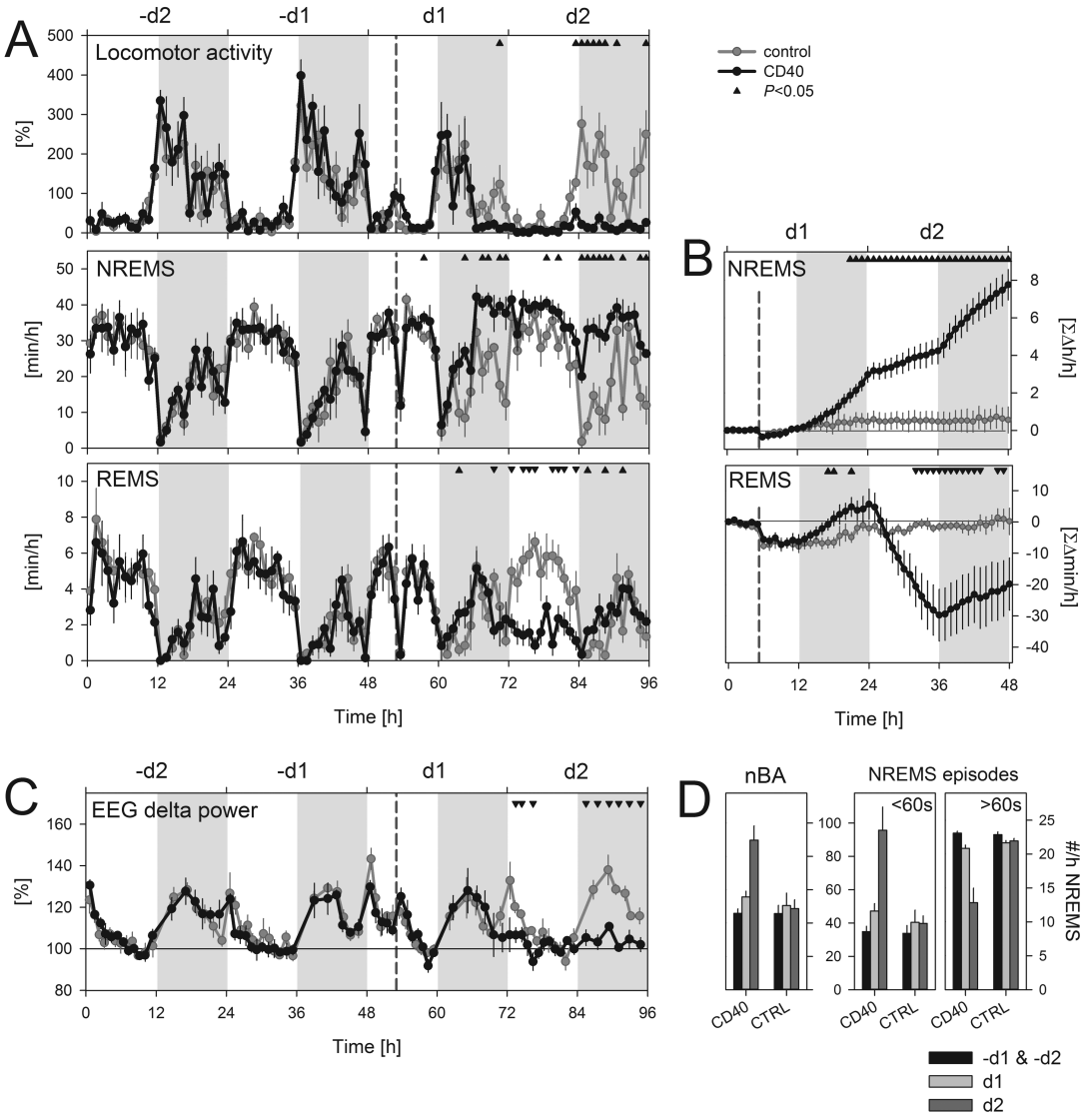

Figure 4: CD40 induced impairment of locomotor activity is associated with enhancement of NREM sleep. (A) Four-day time course of locomotor activity (expressed as % of individual baseline mean), NREM sleep (NREMS), and REM sleep (REMS). Values represent hourly mean values (±1 SEM) during baseline (days -d2 and -d1), day 1 (d1), during which CD40 or IgG2a control was administrated (the time of injection of the antibodies is indicated by dashed vertical lines), and the following day (d2). CD40 suppressed locomotor activity and REMS and promoted NREMS (2-way ANOVA $p < 0.005$ for factors time, treatment, and their interaction; dark triangles mark hourly intervals for which treatment differed; $p < 0.05$, post hoc t-tests, n = 5/treatment). Grey areas delimit the 12 h dark period of the L/D cycle. (B) Analyses of the accumulation of the hourly differences of NREMS and REMS levels reached on d1 and d2 versus individual corresponding baseline values (mean of -d1 and -d2; horizontal line at 0). Dark triangles mark hourly intervals for which treatment differed ($p < 0.05$, t-tests, n = 5/treatment). (C) Time course of EEG delta power during NREMS over the course of the 4-day experiment, and (D) sleep fragmentation quantified as the number of brief

awakenings (nBA) and the number of short and long NREMS episodes (2-way ANOVA $p < 0.05$ for factors time, treatment, and their interaction; dark triangles: day that differed from all other days; $p < 0.05$, post hoc Tukey, n = 5/treatment). Values were expressed as number (#) per h of NREMS to correct for differences in NREMS among days and treatments. Values of -d2 and -d1 were averaged. CD40: CD40 mAb treated mice; CTRL: IgG2a treated mice.

Since etanercept prevents the CD40 mAb induced impairment of locomotor activity (Fig. 3C and D), we assessed the effect of the TNF blocker on the sleep–wake behavior (Fig. 5). The ANOVA showed significant main effects of night-half ($F_{1,18} = 43.057$, $p < 0.001$), and group ($F_{2,18} = 49.762$, $p < 0.001$). Most crucially, all factors significantly interacted to produce individual specific effects ($F_{2,18} = 18.749$, $p < 0.001$). None of the other interactions approached our 0.05 alpha cut-off for significance, although there was a slight trend for the main effect of the measurement-day ($F_{1,18} = 3.990$, $p = 0.061$). Since there was an interaction between all three factors in the ANOVA, we first calculated the differences between the two night halves, and ran individual post hoc tests to determine the source or sources of this significant interaction. Fig. 5 shows significant differences between the CD40 and CD40/

Eta groups in their night-half differences between both days. The CD40 mAb-induced increase of NREM sleep was almost completely averted by the administration of the TNF blocker. This holds true for both dark periods in the 12:12 h L/D cycle, referring to the dark periods of the first and second day after injection of CD40 mAb. Since CD40 mAb significantly inhibited REM sleep in the light period of day 2 (Fig. 4A), we assessed the effect of etanercept at this time point. Etanercept is found to prevent the decrease of REM sleep induced by CD40 mAb almost completely: REM sleep inhibition in the absence and in the presence of etanercept was 54.5% and 9.4% respectively ($p < 0.05$). The difference of REM sleep of 9.4% relative to control animals was not significant. Therefore etanercept blocks the effect of CD40 on REM sleep.

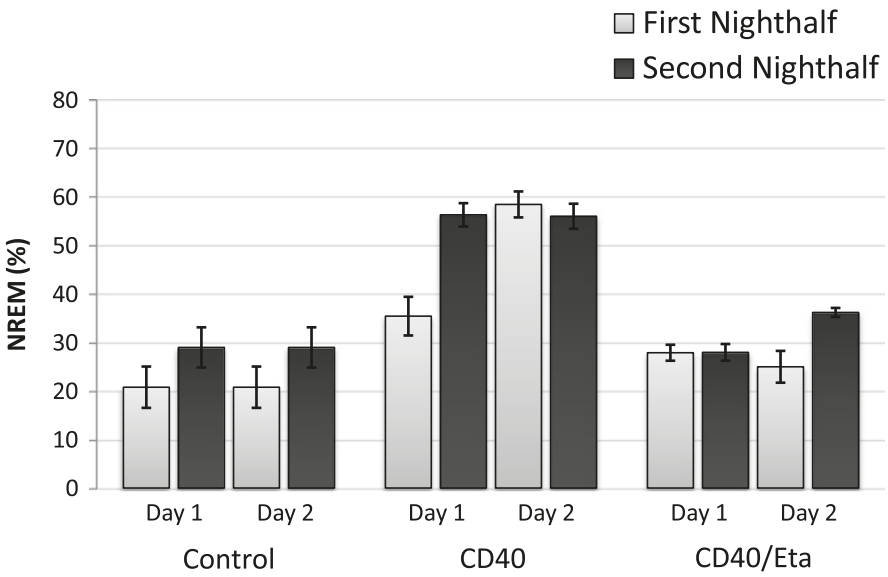

Figure 5: CD40 mAb increase NREM sleep in a TNF dependent pathway. Percentage of NREM sleep during first (1–6 h) and second (7–12 h) night half, split by measurement day. The main group effect is an increase of NREM percentage in the CD40 group ($F_{2,18} = 49.762$, $p < 0.001$). Importantly a three way interaction was found between group, night half and day ($F_{2,18} = 18.749$, $p < 0.001$). Because the main effect found was of night halves ($F_{1,18} = 43.057$, $p < 0.001$) an increase in NREM percentage between the first and second half of the night could be expected. However, there are two exceptions. The first exception is day two of CD40 group ($U6 = 0.000$, $Z = 2.309$, $p = 0.021$) and the second exception is the first day of CD40/Eta ($U6 = 0.000$, $Z = 2.309$, $p = 0.021$) group. ANOVA $p < 0.001$ for factors group (Control, CD40, CD40/Eta); night half (first and second half of the night) and measurement-day (Day1 or Day2); post hoc multiple independent Mann–Whitney U-tests; n = 4 per group). The data are shown as means ± SEM.

CD40 mAb treatment modulates the expression of genes associated with increased homeostatic sleep drive

To test whether CD40 mAb induced NREM sleep is associated with changes in Homer1a expression, total RNA was extracted at day 2 after injection of CD40 mAb from the frontal cortex and cerebellum at ZT9 of the light period and at ZT13 and ZT18 of the dark period. CD40 mAb treatment significantly reduced the expression of Homer1a in the cortex at ZT18, but not at ZT9 and ZT13 (Fig. 6). In control mice, the absolute magnitude of Homer1a expression is low at ZT9, increases at ZT13 and culminates at ZT18 at dark when mice are active, which is in line with previous reports (Maret et al., 2007). This time course contrasts the one observed in CD40 mAb treated mice, in which Homer1a expression decreased from ZT13 to ZT18 (Fig. 6), the time

point of decreased wakefulness and increased NREM sleep. At ZT18 a significant reduction of Egr2, Nptx2, and Fosl2 was also found (Fig. 6). Similar to Homer1a, Egr2 and Fosl2 also decreased from ZT13 to ZT18, the levels of expression reaching those seen at ZT9, the time point of the light period when mice are inactive and have increased NREM sleep (Fig. 6). Compared to control mice, CD40 activation did not lead to altered expression of Jph3 and Ptgs2 at ZT18 (Fig. 6).

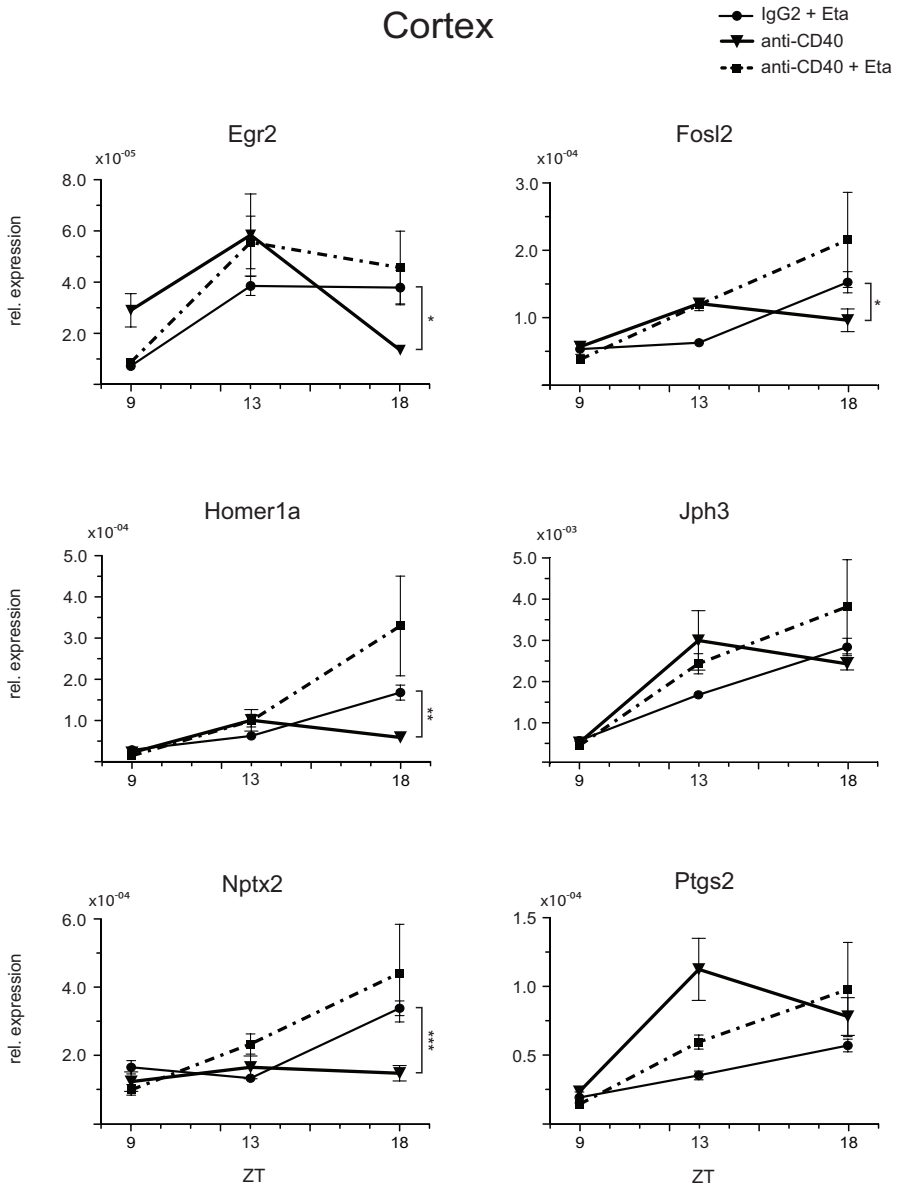

Figure 6: In the second half of the night Homer1a, Egr2, Nptx2 and Fosl2 expression are decreased in the frontal cortex of CD40 mAb treated mice. Mice were treated either with IgG2a and etanercept (Iso + Eta), with anti-CD40 mAb (CD40), or with CD40 mAb and etanercept (CD40 + Eta) and analyzed for gene expression of Egr2, Fosl2, Homer1a, Jph3, Nptx2, and Ptgs2 in the cortex by RT-PCR at ZT9, ZT13 and

ZT18 of day 2 (ZT = 0 lights on, ZT = 12 lights off). Number of mice: ZT9/13/18; IgG-Eta, n = 4/5/5; CD40, n = 5/4/5; CD40-Eta, n = 5/5/6. *p < 0.05, **p < 0.01, ***p < 0.001.

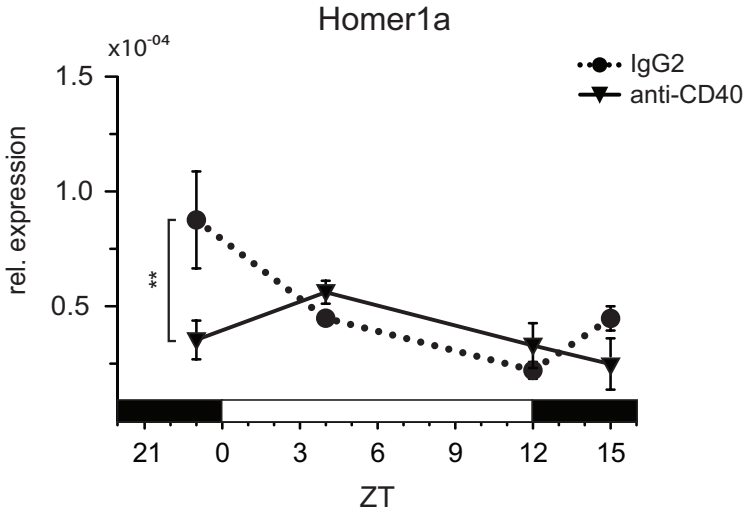

Figure 7: The CD40 mediated decrease of Homer1a expression in the dark period of day2 is seen already in the dark period of day1. Mice were treated with CD40 mAb and the expression of Homer1a assessed on ZT23 of d1 and ZT4, ZT12 and ZT15 of d2. Number of mice per group: 4. ** p < 0.01.

In light of these data, we wondered whether the changes seen in the expression of Homer1a could also be seen at earlier time points after injection of CD40 mAb. Indeed, in CD40 mAb treated mice Homer1a mRNA levels were already decreased in the dark phase of day1 at ZT23 (Fig. 7). Confirming the data outlined above, the experiment showed no difference in Homer1a expression in the light phase of day 2 at ZT3, ZT6 and ZT12. In contrast to the response to CD40 mAb in the cortex, the cerebellum showed no difference of expression of Homer1a, Egr2, Fosl2, Nptx2, and Jph3 at ZT18 of day2 when compared to control mice (data not shown). Ptgs2 was significantly increased, the effect being observed at all three time points tested (data not shown). Since etanercept was shown to interfere with CD40 mAb mediated NREM sleep enhancement, we assessed whether the TNF blocker prevents the CD40 mAb induced modulation of the expression of the aforementioned genes. In etanercept treated mice,

CD40 activation was not associated with decreased expression of Homer1a, Egr2, Nptx2, and Fosl2 at ZT18 (Fig. 6).

We next investigated whether TNF might be involved in the CD40 mAb triggered cascade that alters the expression of the aforementioned genes. Confirming the studies of others, TNF administration is found here to decrease locomotor activity (Table 1). In the hour preceding the end of the experiment at ZT18, the percentage of inhibition of locomotor activity as measured by infrared sensors and recordings of running wheels, was 48% and 61%, respectively. At ZT18, the expression of Homer1a, Egr2, Fosl2, Ptgs2, Jph3, and Nptx2 in the frontal cortex was significantly decreased compared to control mice (Table 1).

Time period	Recording method	Activity change (%)	
ZT12–ZT18	Infrared	−38.29	
	Running Wheel	−52.28	
ZT17–ZT18	Infrared	−47.69	
	Running Wheel	−61.12	
Gene name	Rel. expr.	SEM	p
Homer1a	0.29	0.034	0.0008
Egr2	0.30	0.039	0.0106
Fosl2	0.32	0.036	0.0008
Ptgs2	0.51	0.054	0.0021
Jph3	0.16	0.012	<0.0001
Nptx2	0.46	0.018	0.0003

Table 1: TNF treatment of mice at ZT9 impairs locomotor activity and decreases the expression of genes associated with sleep pressure. Relative expression (rel. expr.) gives the change of expression of the respective genes in the frontal cortex when compared to control mice.

Activation of CD40 increases the expression of Tnf mRNA in the absence of neuroinflammation

NREM sleep enhancement may result from CD40 mediated peripheral inflammation associated with a systemic cytokine response or may be due to CD40 mAb induced CNS inflammation. Histological analysis did not show overt inflammation, endothelial cell disruption, or neuronal cell death in the brain of mice treated for 48 h with CD40 mAb treated mice (n = 5). This was substantiated further by immunohistology. Neither $CD3^+$ T cells nor $B220^+$ B cells and Mac-3^+ monocytes were detected in the vessel wall or

in brain tissues (data not shown). There were no perivascular cuffs and both the meninges and the choroidal plexus of CD40 mAb treated mice did not show T and B cell infiltrates or accumulation of macrophages. Based on Mac-3 and Iba-1 staining, no signs of activation of microglia became apparent (data not shown). In the experiment described here, CD40 mAb triggered immune activation was confirmed by loss of weight (-9.45 ± 1.18 %) and a 4-fold increase in spleen weight. Taken collectively, with the methods used, no evidence was found for CD40 mAb induced neuroinflammation. Because TNF seems to be the critical factor in the CD40 mAb-induced sleep-wake behavior changes, we assessed the site of expression of this cytokine. Tnf mRNA was not only increased significantly in the spleen, but also in the cerebellum and cerebral cortex of CD40 mAb treated mice (Fig. 8A and B). Next we assessed brain TNF levels in tissue homogenates. In pools of supernatants of centrifuged homogenates obtained from frontal cortices and cerebella of IgG treated mice (n = 4), TNF levels were 26.01 and 21.72 pg/mg protein respectively. In pools prepared from CD40 mAb treated mice (n = 4), TNF levels in frontal cortices and cerebella were 19.73 and 18.63 pg/mg protein, respectively. Absence of increase of TNF is not due to interference of tissue factors with TNF in the Luminex-based system. This conclusion is drawn from experiments using supernatants of frontal cortices, which were supplemented with recombinant murine TNF. The level of TNF measured in the samples corresponded to the amount of the cytokine added (data not shown). Further experiments showed TNF protein to be increased in the serum of CD40 mAb treated mice (Fig. 8C). Etanercept, which inhibits binding of TNF to its receptors, significantly reduced the CD40 mAb mediated increase in Tnf expression in the cerebellum, cortex, and liver (data not shown) and abolished the CD40 mAb-mediated increase in serum TNF levels (Fig. 8C).

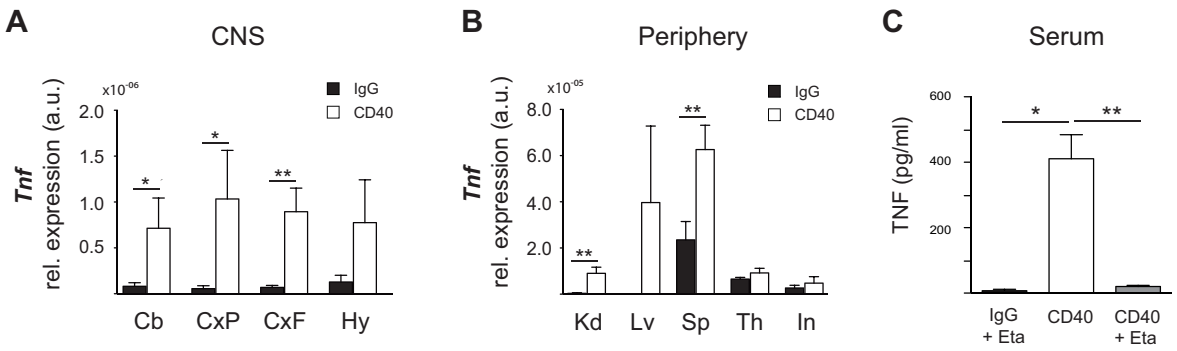

Figure 8: Expression of TNF in the brain and periphery in mice treated with CD40 mAb. The expression of Tnf mRNA in the tissue and TNF in serum were measured at ZT18 of day 1 of injection of CD40 mAb. (A) Tnf gene expression was found to be induced in different regions of the CNS; Cb, Cerebellum; CxP, Cortex parietal; CxF, Cortex frontal; Hy, Hypothalamus. (B) Tnf gene expression is high in liver (Lv), kidney (Kd) and spleen (Sp), but minimal in thymus (Th) and intestine (In). Data are shown as comparative Ct values normalized to 18s rRNA. (n = 3/group; Mann–Whitney U test). (C) Serum TNF induction by CD40 mAb is completely blocked by co-injection of CD40 mAb and etanercept. Number of mice per group: n = 4 for IgG2a-Eta; n = 5 for CD40 mAb and CD40 mAb-Eta. * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

CD40 activation leads to enhancement of NREM sleep with decreased EEG delta power and REM sleep

In the study presented here, we show for the first time that CD40 mAb induced impairment of locomotor activity is associated with altered sleep–wake behavior. Activation of CD40 decreases wakefulness and increases NREM sleep during the dark period. Furthermore, in mice treated with CD40 mAb, EEG delta power during NREM sleep was decreased and did not show variation over time. EEG delta power is known to decrease exponentially as a function of the time spent in NREM sleep (Franken et al., 2001). There is abundant evidence showing that the peripheral or intracerebroventricular injection of cytokines, including TNF and IL-1β, increase NREM sleep (for review see Krueger, 2008, and Opp, 2005). The effects of cytokines on sleep-wake behavior are seen only in the first hours after their application and thus differ considerably from the CD40 mAb-induced, long lasting changes well beyond the 48 h period reported here (data not shown). Prolonged effects are also seen in mice

infected with a mouse-adapted influenza virus. NREM sleep was found to be significantly increased above baseline levels in the dark phase of the second day after infection (Kapás et al., 2008). Opposite to our findings, the NREM sleep increase was not associated with a decrease of REM sleep and of EEG delta power. Thus, NREM sleep enhancement is not always associated with a decrease in EEG delta power. Interestingly when infecting mice lacking both TNF receptors (Tnfr1 and Tnfr2) NREM sleep in the dark phase of d4 and d8 after infection was observed to be associated with a pronounced decrease of EEG delta power (Kapás et al., 2008). Moreover, these changes were also associated with decreased REM sleep in the light phase of the respective days. Thus, at these time points, the effects of infection on sleep resembles those seen in CD40 mAb treated wild type mice with an intact TNF-TNFR1 pathway. As discussed in detail by Kapás et al. (2008), the role of TNF in physiological sleep may differ from effects in conditions of overproduction of TNF. In CD40 mAb-treated mice or in virus-infected mice, other influences including those mediated by monocyte-macrophages and lymphocytes may influence physiological pathways that regulate blood flow, ventilation and metabolism. This aspect is certainly also of relevance in experimental models of LPS-induced disruption of sleep-wake behavior. LPS activates NF-κB, which is a key factor in the production of TNF and IL-1. Thus, LPS-induced changes in sleep architecture may show similarities to the sleep promoting effect of CD40 mAb, which besides of depending on TNF:TNFR1 interactions, is also influenced by activation of IL-1R1 (Taraborrelli et al., 2011). When injecting LPS i.p. into rats, NREM sleep enhancement is completely antagonized by the caspase-1 blocker YVAD, which interferes with cleavage of pro-IL-1 (Imeri and Opp, 2009). Interestingly, YVAD potentiated the effect of LPS regarding REM sleep reduction. This led to the conclusion that IL-1-induced NREM and REM sleep regulation involves different pathways. The hypothesis is supported further in studies on gene double knockout mice, which do not express IL-R1 and TNFR1. These mice spend less time in REM sleep during the light period but show temporally unrelated changes in NREM sleep (Baracchi and Opp, 2008). Here, the CD40 mAb-induced increase of NREM sleep is significantly reduced by etanercept. The TNF blocker also prevented the very pronounced CD40 mAb-mediated decrease of REM sleep, which was only seen in the light phase of day 2. Since at this time point NREM sleep was less affected by CD40 mAb treatment, there is also

evidence for different regulatory mechanisms of NREM and REM sleep in CD40 mAb-mediated sleep-wake changes. The distribution and amount of NREM sleep and the time course of EEG delta power after CD40 administration is reminiscent of that of mice in which circadian rhythms are compromised either by lesion of the suprachiasmatic nucleus (SCN) (Easton et al., 2004) or by deletion of the clock gene Bmal1 (Laposky et al., 2005). In these instances, NREM sleep is increased especially in the dark period, thereby reducing EEG delta power. In contrast to the inhibition of REM sleep in CD40 mAb treated mice, NREM sleep enhancement in Bmal1 gene knockout mice and in SCN-lesioned mice is paralleled by an increase of REM sleep. Based on studies done in mice with a deletion of different clock genes, the current view is that the circadian clock influences homeostatic sleep regulation (Franken and Dijk, 2009). As a hypothesis, the CD40 mAb-induced increase in NREM sleep and resulting decrease of delta power and increased sleep fragmentation may result from a dysregulated expression of clock genes. TNF, the putative mediator of CD40 mAb-induced sleep changes, has been found to suppress the expression of clock genes regulated by E-box DNA motifs (Cavadini et al., 2007; Petrzilka et al., 2009).

On the clinical significance of TNF mediated changes of sleep-wake behavior in CD40 mAb treated mice

Our experiments show that mice treated with the TNF blocker etanercept are resistant to the effects of CD40 mAb to impair locomotor activity in the dark phase, to alter sleep architecture and to prevent increased expression of Homer1a, Egr2, Nptx2, and Fosl2 in the second half of the dark phase of the 12:12 h L/D cycle. In EEG studies of etanercept treated mice the TNF blocker was only used together with CD40 mAb, but has not been added to the IgG2a control. Thus, potential effects of etanercept on physiological sleep escape from being recognized. This is important because mice with deletions of the TNF or TNFR genes as well as mice treated with TNF blocking compounds have been shown to have altered sleep (Kapás et al., 2008). TNF, which is expressed in the CNS of CD40 mAb treated mice, may act on neuronal circuits that are linked to sleep. These data are also interesting from a clinical point of view. Etanercept has been shown to ameliorate SBS in autoimmune diseases. Patients with recent or established rheumatoid arthritis treated with etanercept reported a rapid decrease of fatigue that was sustained

for up to 46 months (Farahani et al., 2006; Franklin, 1998; Moreland et al., 2006). Likewise, etanercept and other TNF blockers also improve quality of life including fatigue in patients with ankylosing spondylitis, inflammatory bowel disease and psoriasis (Braun et al., 2007; Heiberg et al., 2008; Lichtenstein et al., 2002; Tyring et al., 2006). Based on our data, we suggest that patients with autoimmune diseases and sickness behavior syndrome may not only be fatigued, but also excessively sleepy, i.e. show an increased propensity and ability to fall asleep. Excessive daytime sleepiness unrelated to sleep restriction has been described in patients with SLE (Iaboni et al., 2006). The data presented here may also be relevant in the pathogenesis of excessive daytime sleepiness in obstructive sleep apnea (OSA). TNF has been shown to be increased in OSA and etanercept was found to reduce sleepiness in these patients (Vgontzas et al., 2004).

How does etanercept counteract the CD40 mAb induced enhancement of NREM sleep?

The data presented here show that TNF is a downstream mediator of CD40 mAb-induced sleep. TNF may signal to the CNS by activation of the TNFR on the vagus nerve (Kubota et al., 2001) or may cross through brain vessels into the parenchyma. The blood brain barrier (BBB) is a major regulatory interface between the CNS and the peripheral circulation. However, TNF is transported by saturable mechanisms across the BBB. In the context of the data presented here, it is of note that the transport rate (Ki) of TNF in the frontal cortex is significantly lower compared to other sites such as the hypothalamus (Banks et al., 1994). At sites lacking a functional BBB, namely in the perivascular organs, TNF may move from the blood into the parenchyma (Morita and Miyata, 2012). Furthermore, TNF may be produced in the CNS by invading monocytes and dendritic cells. Activation of CD40 has been shown to lead to pronounced inflammation with perivascular and intraparenchymal infiltrates of $CD4^+$ and $CD8^+$ T cells, NK cells, B cells, neutrophils, dendritic cells and macrophages in the lungs, liver, intestinal tract and pancreas (Kimura et al., 2006; Wiley and Harmsen, 1999). In striking contrast, in the CNS our data show that CD40 mAb treatment does not lead to an inflammatory response. This finding is unexpected because brain endothelial cells express CD40 and respond to CD40 activation in vitro with increased expression of adhesion molecules and transmigration of monocytes (Ramirez et al., 2010; Vowinkel et

al., 2006). By RT-PCR analysis, we show Tnf expression to be increased in the brain of CD40 mAb treated mice. However, at the protein level we failed to demonstrate a CD40 mAb-induced increase of TNF in the frontal cortex and cerebellum. In agreement with previous reports, the methods used allow detection of TNF in brain homogenates of mice. Intraperitoneal injection of LPS (10 µl) leads to a small, but statistically significant 2-fold increase of TNF in the hypothalamus, hippocampus and brain stem (Datta and Opp, 2008). Comparable data have been obtained recently by Erickson and Banks (2011). CD40 mediated effects on TNF mRNA, but not TNF protein may be due to activation induced posttranscriptional or posttranslational events. Transmembrane TNF, the bioactive membrane-anchored precursor of the soluble form of TNF, is processed by TNF-converting enzyme (TACE/ADAM17), which leads to cleavage and release of the soluble form of TNF (Horiuchi et al., 2010). With the method used, the membrane form of TNF may escape from recognition because, at least in part, cell membranes are removed by the centrifugation process. Furthermore, since TNF on the cell membrane undergoes rapid endocytosis and degradation, turnover of the cytokine may depend on its cellular source and the condition leading to cell activation (Shurety et al., 2001). Thus, low TNF levels after LPS administration or undetectable increases of the cytokine after CD40 mAb treatment does not exclude the presence and function of TNF at distinct sites in the brain. The absence of both inflammation in the CNS tissue and signs of activation of microglia may indicate that TNF mRNA is expressed by brain endothelial cells. In a first step, endothelial cells may produce TNF upon stimulation of CD40 (Song et al., 2011). Thereafter TNF, which is found here to be highly increased in serum of mice treated with CD40 mAb, may enhance its own transcription. This hypothesis is based on the following findings: (1) TNF upregulates IκB mRNA (inhibitor of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) in cerebral endothelial cells; (2) the TNF promoter harbors a NF-κB (nuclear factor 'kappa-light-chain enhancer' of activated B-cells) site and is regulated by the interplay of IκB and NFκB; (3) the systemic treatment of mice with TNF induces TNF mRNA and protein as well as NFκB p65 in the brain (Laflamme and Rivest, 1999; Qin et al., 2007). The finding of low TNF serum concentrations and Tnf mRNA expression in the CNS of mice treated with CD40 mAb in the presence of etanercept may be explained by etanercept-

induced interference with the TNF amplification pathway outlined here. It remains unclear on what type of cell TNFR1 expression is required for CD40 mediated SBS.

Decreased expression of Homer1a, Egr2, Nptx2 and Fosl2 in CD40 mAb treated mice is associated with NREM sleep enhancement

At the molecular level, the accumulation of sleep need during wakefulness and the discharge of sleep need during sleep are little understood, but are likely to involve changes in gene expression. Genetic studies and transcriptome profiling identified Homer1a as a core molecular correlate of the homeostatic sleep process (Franken et al., 2001; Maret et al., 2007). In our study, Homer1a expression in the cortex differs considerably when comparing

CD40 mAb treated mice with control mice. In the dark phase of the 12:12 h L/D cycle Homer1a expression in the cortex of control mice was found to increase with time spent awake. CD40 activation prevents the progressive increase of expression of Homer1a during the second half of the dark period. Thus, enhancement of NREM sleep is associated with decreased expression of Homer1a. Homer1a expression did not differ during the light phase, in which CD40 mAb led to a striking decrease of REM sleep. As a hypothesis, the effect of CD40 activation on Homer1a expression in the dark phase may influence sleep architecture in the following light phase. Homer1a mRNA, which is a short splice variant of the Homer1 gene, increases during spontaneous and enforced periods of wakefulness (Huber et al., 2007b; Maret et al., 2007; Nelson et al., 2004). In the context of the data presented here it is highly interesting that C57BL/6J mice with a deletion of the Homer H1a splice form of the Homer1 gene (Homer1a KO mice) show reduced wakefulness with increased NREM and REM sleep during the dark period (Naidoo et al., 2012). The effect of the deletion of Homer1a on sleep architecture was accompanied by a decrease of REM sleep in the light phase, which, however, did not reach statistical significance. Taken together, the effect of CD40 mAb-induced, TNF-dependent decrease of expression of Homer1a during wakefulness may play a pivotal role in the regulation of sleep-wake behavior. The effect of the immune system on sleep-wake behavior may also include altered expression of other genes that have been described to be changed in experiments using sleep deprivation, including Egr2, Fosl2, Nptx2, Ptgs2, and Jph3 (Maret et al., 2007). In the cortex of CD40 mAb

treated mice, the decline of Homer1a from ZT13 to ZT18 was associated with a significant depression of Egr2, Nptx2, and Fosl2. Our data also show that the effect of CD40 mAb on the above-mentioned genes is prevented by the administration of etanercept. In line with these experiments, i.p. injection of TNF leads to a significant reduction of Homer1a, Egr2, Fosl2, Nptx2, Ptgs2, and Jph3 expression in the cortex at ZT18, the time point when TNF-treated mice have decreased locomotor activity. Taken collectively, our data show that TNF plays an essential role in the effect of CD40 on sleep-wake behavior and on the expression of genes linked to sleep homeostasis.

CONCLUSION

TNF:TNFR interactions play a pivotal role in sleep regulatory circuits (Krueger, 2008). This may be fundamental in infectious diseases, as sleep is considered to play a protective and regenerative role. However, in patients with autoimmune diseases, the immune response to self-antigens may, through CD40-mediated production of TNF, interfere with regulatory systems responsible for sleep-wake regulation, resulting in pathologically increased sleep and sleep propensity.

Author contributions

H.G., A.M., T.B., Y.E., and P.F. designed and performed the experiments. M.L., D.M., R.H., F.D., M.P., and A.F. contributed to the concept of the study.

Conflict of Interest Statement

All authors declare that there are no conflicts of interest.

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3.3 Tumor necrosis factor and transforming growth factor beta regulate clock genes by controlling the expression of the cold inducible RNA-binding protein (CIRBP)

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ABSTRACT

The circadian clock drives the rhythmic expression of a broad array of genes that orchestrate metabolism, sleep wake behavior and the immune response. Clock genes are transcriptional regulators engaged in the generation of circadian rhythms. The cold inducible RNA-binding protein (CIRBP) guarantees high amplitude expression of clock. The cytokines TNF and TGFβ impair the expression of clock genes, namely the period genes and the proline- and acidic amino acid-rich basic leucine zipper (PAR-bZip) clock-controlled genes. Here, we show that TNF and TGFβ impair the expression of *Cirbp* in fibroblasts and neuronal cells. IL-1β, IL-6, IFNα, and IFNγ do not exert such effects. Depletion of *Cirbp* is found to increase the susceptibility of cells to the TNF-mediated inhibition of high amplitude expression of clock genes and modulates the TNF-induced cytokine response. Our findings reveal a new mechanism of cytokine-regulated expression of clock genes.

INTRODUCTION

Clock genes mediate circadian rhythmicity and thereby control mammalian metabolism and sleep-wake behavior (Eckel-Mahan and Sassone-Corsi, 2012; Franken and Dijk, 2009). The heterodimerized transcription factor CLOCK:BMAL1 (brain and muscle ARNT–like protein) activates transcription by binding to E-box motives of *Period (Per)*, *Cryptochrome (Cry)* and the following members of the PAR-bZip family of transcription factor genes: the *D-site albumin promoter binding protein* (*Dbp*), *Tyrotroph embryonic factor* (*Tef*) and *Hepatic leukemia factor* (*Hlf*) (Dibner et al.; Ko and Takahashi, 2006). PER and CRY proteins inhibit the function of CLOCK:BMAL1 complexes, thereby inhibiting their own gene expression. This feedback-loop mechanism generates circadian oscillations of *Per (Per1, Per2, Per3)* and *Cry (Cry1, Cry2)* expression. The same positive and negative regulatory components also govern the rhythmic expression of the nuclear orphan receptor *Rev-Erbα*, which in turn represses the transcription of *Bmal1* through direct binding to a REV-ERBα response element in the *Bmal1* promoter. Thereby, REV-ERBα interconnects the cyclic expression of positive- and negative-loop members. These pathways drive the 24hr rhythms in physiology, behavior and the immune response to microbes. The crosstalk between the clock system and the immune system has been addressed in several recent studies. The extent of secretion of TNF and IL-6 by LPS stimulated macrophages follows a circadian rhythm (Keller et al., 2009). The macrophage response to LPS is decreased in mice with an inactivation of the *Clock* gene (Bellet et al., 2013). Compared to bone marrow derived macrophages obtained from wild type (WT) mice, the expression of cytokines including IL-1β, IL-6, TNF, IFNα and IFNγ was significantly lower in clock mutant mice. Inactivation of the clock genes Cry1 and Cry2 leads to increased production of TNF, IL-1β and IL-6 in experimental arthritis (Hashiramoto et al., 2010). Per2 gene knockout mice show a defective natural killer cell function with decreased IFNγ and IL-1i serum concentrations following LPS challenge (Liu et al., 2006). A nonfunctional Per2 gene also affects TLR9 expression and thereby the vaccine response when using TLR9 ligands as adjuvant (Silver et al., 2012b). Taken collectively, the data show that the circadian clock controls immune responsiveness.

A compelling recent study shed light on post-transcriptional regulation required for circadian orchestration of the clock network. Loss-of-function experiments indicate that

the cold-inducible RNA binding protein (CIRBP) is required for high amplitude circadian gene expression (Morf et al., 2012). The RNA binding protein CIRBP belongs to the highly conserved glycin-rich RNA binding protein family and is thought to modulate gene expression by binding to transcripts in the 5`- untranslated region (UTR) or 3`- UTR of mRNA and thereby affect the rate of translation initiation and stability of the transcript (Fujita, 1999; Liu et al., 2012; Morf et al., 2012). *Cirbp* is constitutively expressed mainly in testis, lung, heart, kidney, hippocampus and cerebral cortex of the adult rat (Nishiyama et al., 1998). The expression of *Cirbp* mRNA and protein is upregulated by cellular stress, including mild cold stress (32°C) and hypoxic conditions (LLeonart, 2009). In hypoxia CIRBP is released from lysosomes of macrophages and is found in the serum of patients with shock due to hemorrhages or trauma (Qiang et al., 2013). Surprisingly CIRBP is found to bind to the LPS receptor TLR4, thereby activating the release of proinflammatory cytokines including TNF and IL-6. These data point to a pivotal role of CIRBP in the innate immune response.

CIRBP may serve as a chaperone that assists in the folding / unfolding, assembly / disassembly and transport of various proteins (Fujita, 1999; Gualerzi et al., 2003). High-amplitude expression of transcripts associated with circadian clock function has been found to depend on CIRBP. Among the transcripts interacting with CIRBP are the mRNAs encoding Sirtuin-1 (SIRT1), PER2, PER3 and DBP, which were decreased in *Cirbp* depleted cells (Liu et al., 2012; Morf et al., 2012). The phenotype caused by *Cirbp* deficiency resembles most closely that observed in cells depleted of *Clock*. In both *Cirbp* and *Clock* depleted cells, amounts of *Bmal1* mRNA were increased, whereas *Per3, Per2,* and *Dbp* mRNA were decreased (Morf et al., 2012). This phenotype is also reminiscent of the pattern of clock gene expression described in TNF and TGFβ treated cells. These cytokines have been shown recently to lead to inhibition of expression of *Per1*, *Per2*, and *Per3* and of the PAR-bZip clock-controlled genes *Dbp, Tef*, and *Hlf* and to upregulation of *Bmal1* (Cavadini et al., 2007; Gast et al., 2012). Dysregulation of clock gene expression by cytokines has been described in fibroblasts, human pancreas cancer cells and in leukocytes *in vitro*, and in the liver of mice treated with TNF or LPS (Haimovich et al., 2010; Ohdo et al., 2001; Okada et al., 2008; Suzuki et al., 2008).

The molecular mechanisms, which lead to cytokine-induced dysregulation of expression of clock genes, are not yet clear. Using reporter genes, TNF was found to

inhibit CLOCK:BMAL1-induced activation of E-box regulatory elements in clock gene promoters (Cavadini et al., 2007). Since the RNA binding Protein CIRBP was previously described to be required for high amplitude circadian gene expression, we explored the hypothesis that cytokines may interfere with *Cirbp* expression. Our data show that TNF and TGFβ inhibit the production of *Cirbp* and thereby enhance the suppressive effect of the cytokines on clock genes. Moreover downregulation of *Cirbp* expression is found to have an impact on basal and TNF stimulated cytokine expression.

EXPERIMENTAL PROCEDURES

Treatment of cells with cytokines and blockers.

NIH-3T3 fibroblasts, HT22 neuronal cells, C57BL/6 WT mouse embryonic fibroblasts (MEF) and *Cirbp*$^{-/-}$ MEFs were grown in DMEM high glucose (4,5g/l) medium supplemented with 10% FCS and penicillin/streptomycin (1x). Cells were kept at 37°C and 5% CO_2. For RT-qPCR analysis, cells were seeded in triplicates in 12-well plates in a density of $1x10^5$ per well. For Western Blots cells were seed in T25 flasks in a density of $1x10^6$ per flask. Two days after seeding, cells were synchronized by serum deprivation (1% FCS) for 1hr and then treated with murine cytokines. TNF, TGFβ, IL-1β, IL-6 and IFNγ were obtained from Peprotech and IFNα from Miltenyi Biotech.

Treatment of cells with siRNA against Cirbp.

NIH-3T3 cells were transfected with DharmaFECT 1 transfection reagent (Dharmacon) and 25nM of siRNA (Dharmacon) against *Cirbp*. *Gapdh* and no target siRNA were used as positive and negative controls respectively. After transfection cells were incubated for 36hr for RNA analysis and 48hr for protein analysis.

RNA isolation and gene expression analysis.

Whole-cell RNA from cultured cells was extracted using the NucleoSpin RNA II kit (Macherey-Nagel). Subsequently, RNA was reverse-transcribed using random hexamers (Fermentas) and M-MuLV reverse transcriptase (Roche). One μg of total

RNA was amplified in an ABI 7900 HT detection system (Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems). Sequences for custom made *Cirbp* TaqMan Assays see Table 1. The relative levels of each RNA were calculated by the $2^{-\Delta\Delta CT}$ method; *eEF1a1* and *GAPDH* were used as housekeeping genes. Each CT value used for these calculations is the mean of triplicates of the same reaction. Relative RNA levels are expressed as x-fold variations compared to ZT = 0 (time course experiments) or as percentages of the average control groups. RNA stability measurements were performed by using 5µg/ml of the transcription blocker Actinomycin D (ActD) (Sigma). The compound was added to the cultures 2hr after starting cytokine treatment (see above). Cells were harvested at the indicated time points and the amount of extracted RNA was analyzed with qPCR after reverse transcription.

Table 1: Sequences for custom made *Cirbp* TaqMan Assays

Primer forward 5'>3':	CAGATCTCCGAAGTGGTGGT
Primer reverse 5'>3:	CCAGCCTGGTCAACTCTGAT
Probe:	GGCTTTGGGTTTGTCACCTTTG

Cytokine and chemokine array.

Expression of cytokine and chemokine genes was tested by using a StellARray assay (Bar Harbor Biotechnology) for the expression of immunology related genes in mice. The array was performed accordingly to the manufactures instructions.

Analysis of CIRP by Western Blot.

Cells were lysed with the IP lysis buffer (Pierce) as described in the protocol. Whole protein extracts (40µg) in LDS sample buffer (Invitrogen) and DTT were applied on a NuPAGE 12% Bis-Tris-Gel (Invitrogen). The proteins were separated at constant 150V in a MES SDS running buffer (Invitrogen). Subsequently blotting on a PVDF membrane was performed in a full wet tank blot. Membranes were incubated with a CIRBP rabbit polyclonal antibody recognizing the C terminus of mouse CIRBP (Masuda et al., 2012). As secondary antibody an HRP-conjugated goat anti-rabbit (ab79051, Abcam) was used and incubated for 1hr. As a loading control an antibody to the mouse nuclear matrix

protein p84 was used (1hr, ab487). The secondary antibody used with the anti-p84 was a goat to mouse HRP (1hr, ab97023). For densitometric measurements the Western Blots were analyzed with the ImageJ Software. The relative values were normalized to the loading controls.

Cirbp complementation and overexpression.

Cirbp$^{-/-}$ MEFs (24) were grown as described above and transiently transfected with a *Cirbp* overexpressing plasmid. Cells (8x10^5 per T-25 flask) were seeded and transfected with 8.8µg of a pCMV6::*Cirbp* expression vector (Origene) and FugeneHD transfection reagent (Promega). 48hr later cells were treated with TNF (10ng/ml) or left untreated as control. Using the same procedure, WT MEFs were transfected with the pCMV6::*Cirbp* plasmid to generate a stable *Cirbp* overexpressing cell line.

Statistics.

Graphs are plotted as a mean of triplicates with S.E.M. Students t test was performed with PRISM software.

RESULTS

TNF and TGFβ inhibit the expression of CIRBP.

The effect of cytokines on *Cirbp* expression was assessed in NIH-3T3 fibroblasts and HT22 neuronal cells. The individual cytokines were tested in concentrations, which have been described to mediate maximal bioactivity in their standard assays including studies on clock gene expression (Cavadini et al., 2007). In NIH-3T3 cells TNF decreases *Cirbp* mRNA expression by 50% (**Figure 1A**). A pronounced reduction of *Cirbp* mRNA was also observed with TGFβ and to a lesser extent with IL-1β (inhibition 22%). Neither IL-6 nor IFNγ affected *Cirbp* mRNA expression. The inhibitory effect of IFNα was significant, the extent of inhibition, however, being only 15%. Downregulation of *Cirbp* mRNA was also seen when treating HT22 hippocampal neurons with TNF and TGFβ, maximal inhibition being 37% and 22% respectively (**Figure 1B**). In HT22 cells IL-1β, IL-6, IFNα and IFNγ failed to alter *Cirbp* expression.

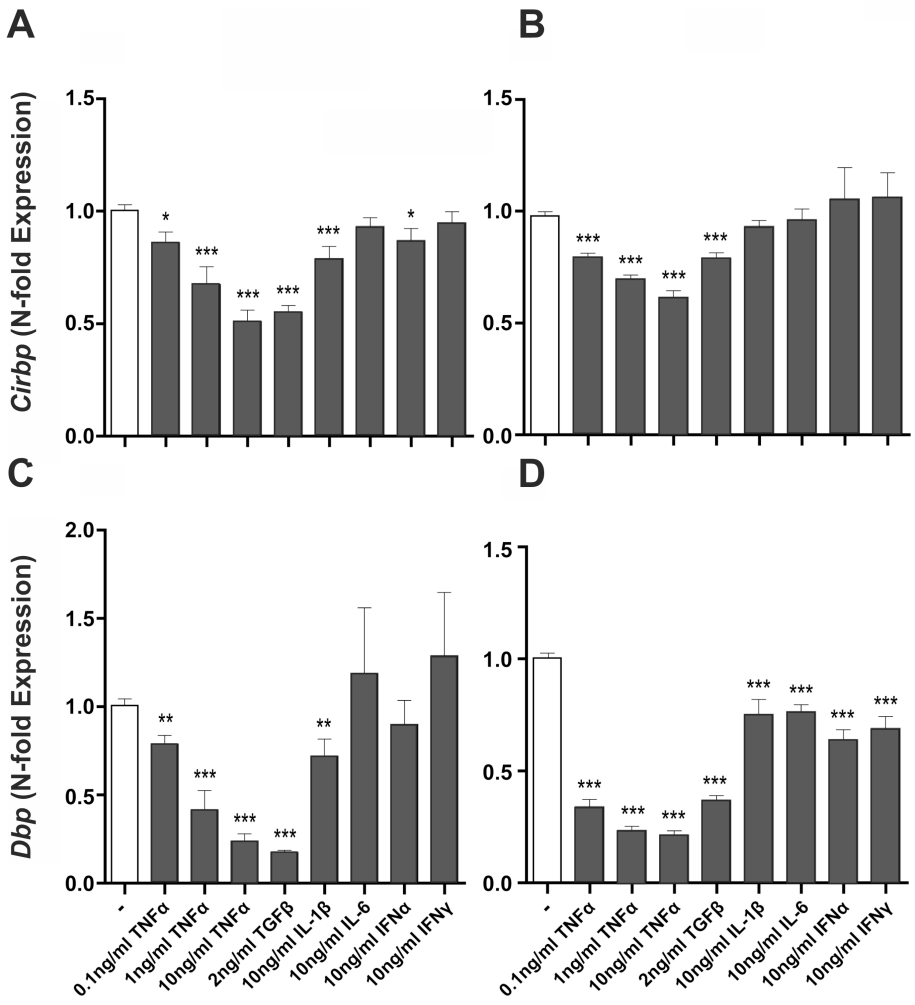


Figure 1: TNF and TGFβ inhibit the expression of *Cirbp* (A and B) and of *Dbp* (C and D) mRNA in NIH-3T3 (A and C) and HT22 cells (B and D) exposed for 4hr to cytokines (grey bars) (untreated control: white bars). Data of RT-qPCR assays of *Cirbp* and *Dbp* expression show the mean S.E.M of triplicates from one representative experiment of three; independent t test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

To assess the relationship between the expression of *Cirbp* and clock genes, we analyzed the expression level of *Dbp* mRNA. Confirming recent studies TNF and TGFβ inhibit *Dbp* mRNA expression in NIH-3T3 cells (**Figure 1C**). While the effect of IL-1β was much less pronounced, no effects on *Dbp* expression were seen when treating the cells with IL-6, IFNα or IFNγ. When treating NIH-3T3 cells with both TNF and TGFβ, the effect of low dose TNF on *Dbp* mRNA expression was increased significantly by TGFβ. When testing HT22 cells *Dbp* mRNA expression was found to be profoundly inhibited by TNF and TGFβ (**Figure 1D**). Whereas *Cirbp* mRNA expression in neuronal cells treated with IL-1β, IL-6, IFNα and IFNγ was not altered, the cytokines decreased

Dbp mRNA expression the percentages of inhibition being 25%, 24%, 37% and 32% respectively (**Figure 1D**). Taken collectively, the most efficient interference with the expression of both *Cirbp* and *Dbp* mRNA in NIH-3T3 as well as in HT22 cells is seen with TNF and TGFβ. However, the concomitant treatment of the cells with both cytokines did not show additive effects of TGFβ on the TNF induced inhibition of *Cirbp* expression (**Figure 2**). Since *Cirbp* appears to be regulated at two levels, the first at the transcriptional level and the second at the translational level (Al-Fageeh and Smales, 2009), we studied the effect of cytokines also on CIRBP protein levels. Western Blot analysis of cell lysates of TNF and TGFβ treated NIH-3T3 cells showed a pronounced reduction of the amount of CIRBP **(Figure 3)**. This contrasts IL-1β that only slightly decreased *Cirbp* mRNA, and had only minimal effects on CIRBP protein expression (**Figure 3**).

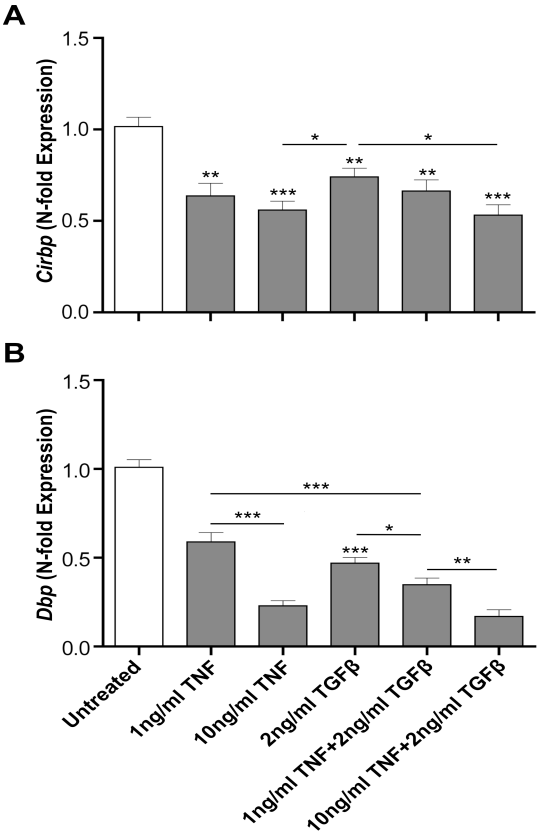
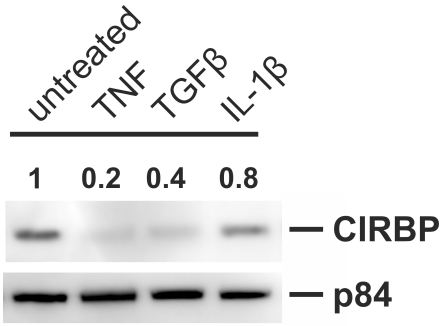


Figure 2 (left): Effects on the expression of *Cirbp* (A) and *Dbp* (B) after the co-treatment of NIH-3T3 cells with TNF and TGFβ. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 3 (below): Western Blot analysis of CIRBP in NIH-3T3 cells treated for 4hr with TNF (10ng/ml), TGFβ (2ng/ml) and IL1-β (10ng/ml). As a loading control p84 was used. Numerics indicate the densitometric ratio comparing the bands *to the untreated control.*



Cirbp has been described to show circadian oscillations in the adult mouse brain (Nishiyama et al., 1999). Circadian rhythms of expression have also been observed in the liver with *Cirbp* mRNA levels peaking at Zeitgeber times when body temperature was minimal(Kornmann et al., 2007). We found that also in NIH-3T3 cells *Cirbp* expression was rhythmic over a 23hr study period (**Figure 4A**). A robust circadian expression of *Cirbp* mRNA was also seen in TNF treated cultures (**Figure 4A**). The phase of the rhythm was not affected by TNF. However, the amplitude of expression of *Cirbp* mRNA was significantly lower, the extent of decreased expression being comparable at all-time points tested. At the protein level TNF inhibited CIRBP in both HT22 and NIH-3T3 cells treated for 8hr and 24hr with the cytokine (**Figure 4B**). The effect of TGFβ was transient. The cytokine failed to inhibit CIRBP expression in 3T3 cells exposed for time periods, which exceed 4hr. In HT22 cells TGFβ was found to have effects at 8hr, but not at 24hr. Taken collectively, the most uniform effects were observed with TNF which inhibited the expression of CIRBP in both fibroblasts and neuronal cells over a prolonged time period.

Morf et al. (2012) observed rhythmic *Cirbp* expression in NIH-3T3 cells only upon exposure to temperature rhythms. In the aforementioned study synchronization of the cultured cells was performed by serum shock rather than serum deprivation, the method being used here. Indeed, when adapting our method and adding 50% horse serum *Cirbp* mRNA did not show a rhythmic expression. However, TNF also decreased *Cirbp* expression in this culture condition.

CIRBP belongs to the family of RNA binding proteins and thereby may modulate mRNA stability. We examined if treatment of cells with TNF alters the decay of *Dbp* mRNA after adding the transcription blocker ActD. A 2hr pretreatment with TNF before adding ActD did not alter the decline of *Dbp* mRNA over time when compared to untreated cells (**Figure. 4C**). Moreover, the decline of *Dbp* mRNA was not different in *Cirbp*$^{-/-}$ MEFs compared to WT MEFs. These data do not support the hypothesis that diminution of *Cirbp* by TNF destabilizes *Dbp* mRNA and thereby prevents from its high amplitude expression. When investigating the effect of TNF on the stability of *Cirbp* mRNA following ActD treatment, the cytokine was not found to influence the decline of the *Cirbp* mRNA (**Figure 4D**).

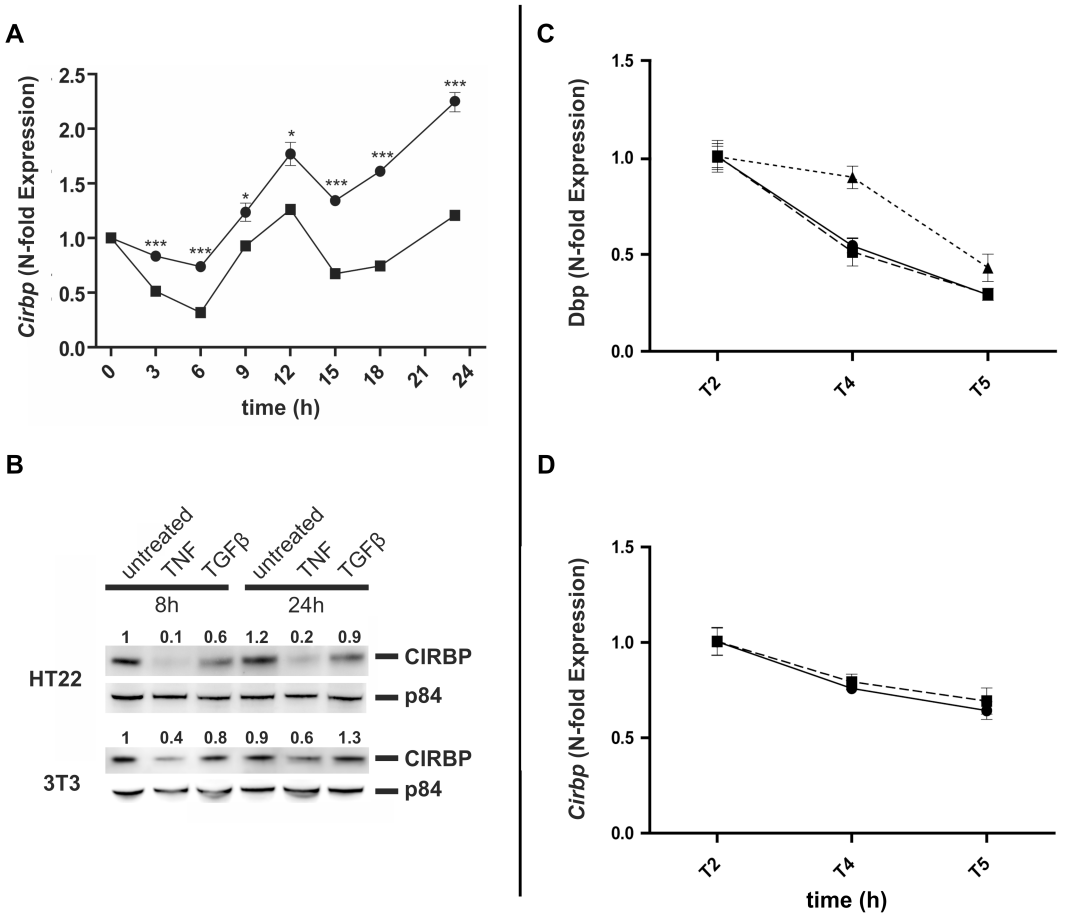


Figure 4: Time kinetic experiments and mRNA stability. (A) Time course experiments of *Cirbp* mRNA expression in MEFs cells treated with TNF (10ng/ml) (black squares) or media control (black circles). In TNF treated cultures a significant reduction of the circadian expression of *Cirbp* mRNA is seen at all-time points analyzed. (B) Western Blot of TNF (10ng/ml) and TGFβ (2ng/ml) treated HT22 and NIH-3T3 cells, the cultures being exposed to the cytokines for 8hr and 24hr. P84 was used as a loading control. *Dbp* (C) and *Cirbp* (D) mRNA stability of untreated MEFs (black circles) and TNF (10ng/ml) treated cells (black squares). Data of Cirbp$^{-/-}$ MEFs (untreated) are shown in black triangles. After one hour of serum deprivation, at T2 ActD (5µg/ml) was added and the WT MEFs analyzed at the indicated time points with qPCR.

The cytokine induced inhibition of the production of CIRBP correlates with their effects on clock gene expression.

A relationship of the effects of TNF on the expression of *Cirbp* and *Dbp* is supported when analyzing the kinetics of the expression of the respective genes. Whereas in NIH-3T3 cells *Cirbp* mRNA and *Dbp* mRNA were not altered by treatment of the cells with

TNF for 30 min or 60 min; a robust inhibition of the expression of both genes was seen at 120 min and 240 min respectively (**Figure 5A and 5B**).

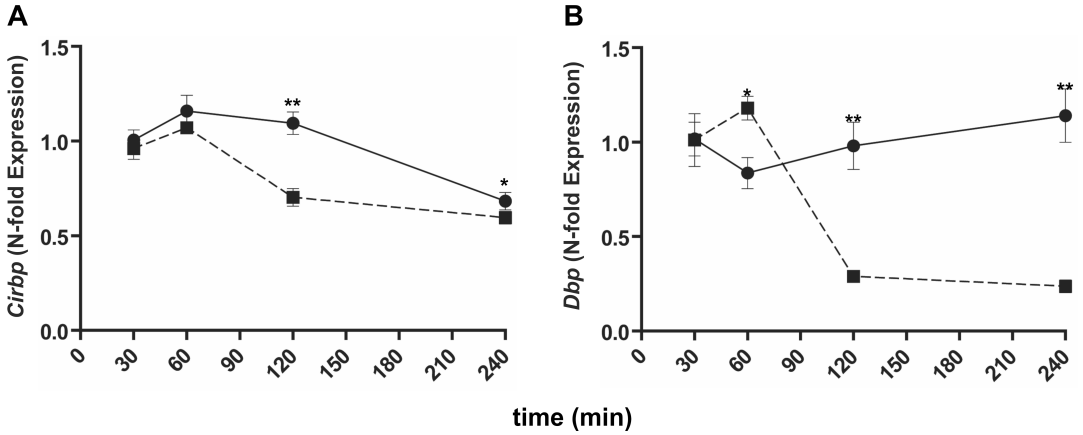


Figure 5: Time dependent expression of *Cirbp* (A) and *Dbp* (B) mRNA in TNF treated NIH-3T3 cells**.** Cultures were treated with TNF (10 ng/ml) (broken line) or PBS control (closed line). RNA was harvested at various time points after treatment. One representative experiment of three; mean values of triplicates S.E.M; independent t test * $p < 0.05$, ** $p < 0.01$.

Silencing of Cirbp with siRNA enhances TNF and TGFβ-induced suppression of Dbp mRNA.

Because CIRBP enables high amplitude expression of clock genes including *Dbp* (Morf et al., 2012) and because, as shown here, TNF and TGFβ suppresses both *Cirbp* and *Dbp* expression, we determined whether the effect of TNF and TGFβ on *Dbp* is mediated through CIRBP. This hypothesis was tested by suppressing *Cirbp* expression using siRNA. NIH-3T3 cells were transfected for 36hr with siRNA against *Cirbp* and siNoTarget control. Thereafter, cells were treated with TNF, which was added in a high or low dose concentration. TNF added in a high dose (10ng/ml) reduced both *Cirbp* and *Dbp* mRNA (**Figure 6A and 6B**). The expression of *Cirbp* and of *Dbp* was less affected when treating MEFs with a low TNF dose. However, the depletion of *Cirbp* by siRNA made the cells to become significantly more responsive to the effect of low dose TNF to inhibit *Dbp* expression (**Figure 6B**). While in the absence of *Cirbp* depletion the TNF mediated inhibition of *Dbp* expression was 35%, the respective value in cells treated with siRNA against *Cirbp* was 69% ($p < 0.01$). Treatment of *Cirbp* depleted and non-depleted cells with the high dose of TNF, was followed by an inhibition of *Dbp*

expression of 91% and 89% respectively. Analogous experiments with TGFβ show that also the inhibitory effect of TGFβ on *Dbp* mRNA expression was more pronounced in cells treated with siRNA against *Cirbp* compared to control cells (37% versus 46%). However, the difference did not reach statistical significance (**Figure 6C and 6D**). Western Blot analysis confirmed the knockdown of *Cirbp* by siRNA, the effect being enhanced by TNF high dose (**Figure 7**).

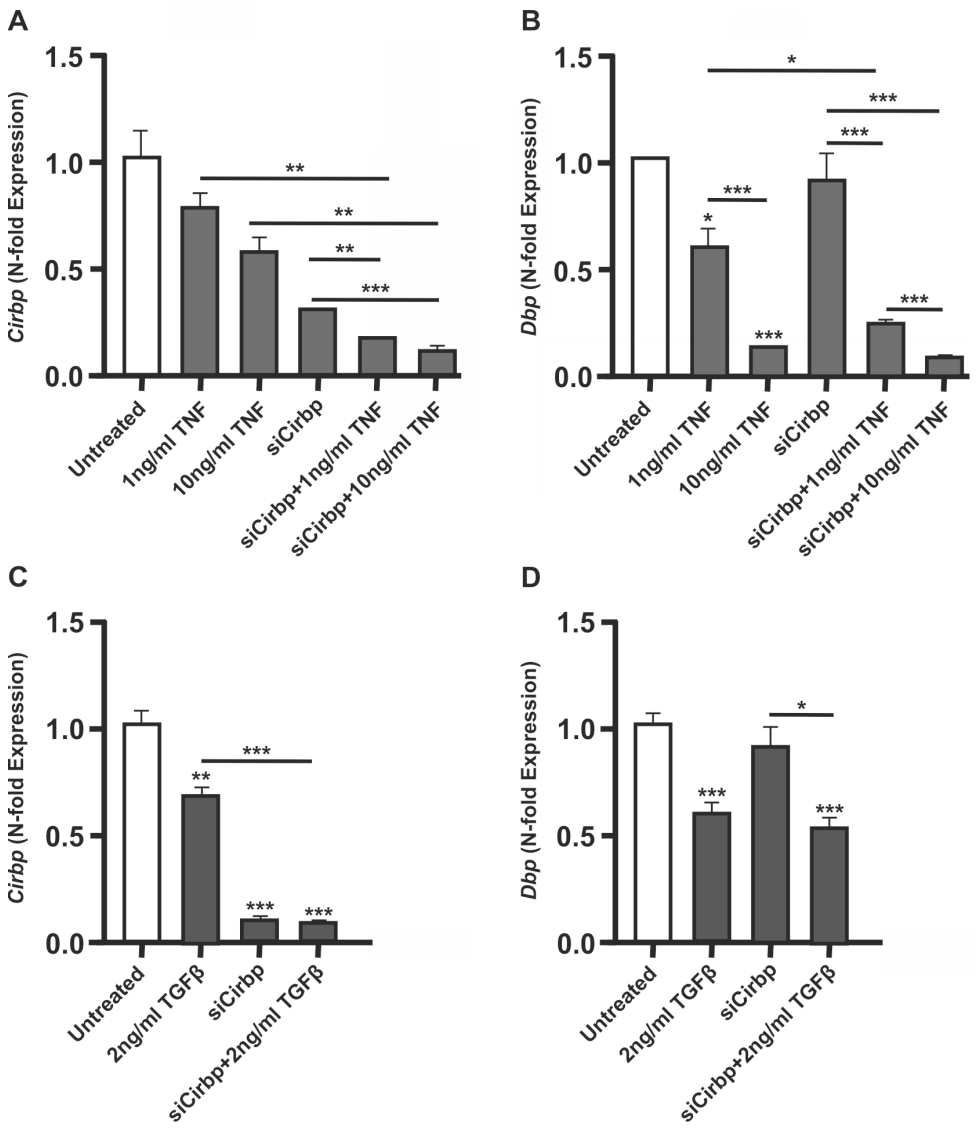


Figure 6: The expression of *Cirbp* and *Dbp* mRNA was tested in *Cirbp* depleted cells that were treated with TNF (A and B) and TGFβ (C and D). The data show the effect of cytokines and of siRNA against *Cirbp* and *Dbp* that were added to the cultures alone or in combination. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

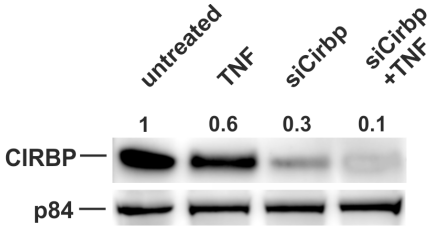


Figure 7: Control Western Blot to show the effective repression of CIRBP by downregulating *Cirbp* with siRNA. P84 was used as a loading control; the digits show the ratio from the protein of interest to the loading control.

Cells with an inactivation of the Cirbp gene respond to TNF with increased inhibition of clock genes.

Since we found that siRNA against *Cirbp* sensitizes the cells to the effect of TNF to suppress *Dbp* mRNA, we analyzed the expression of clock genes in (MEFs), which were established from mice with an inactivation of the *Cirbp* gene (*Cirbp*$^{-/-}$ MEFs) (Masuda et al., 2012). Compared to WT MEFs the effect of TNF to lower the expression of clock genes was found to be more pronounced in *Cirbp*$^{-/-}$ MEFs (**Figure 8**). Upon treatment with TNF the expression of *Per3* and the PAR-bZip clock controlled genes *Dbp, Hlf,* and *Tef* was significantly lower in *Cirbp*$^{-/-}$ MEFs then in TNF treated control MEFs.

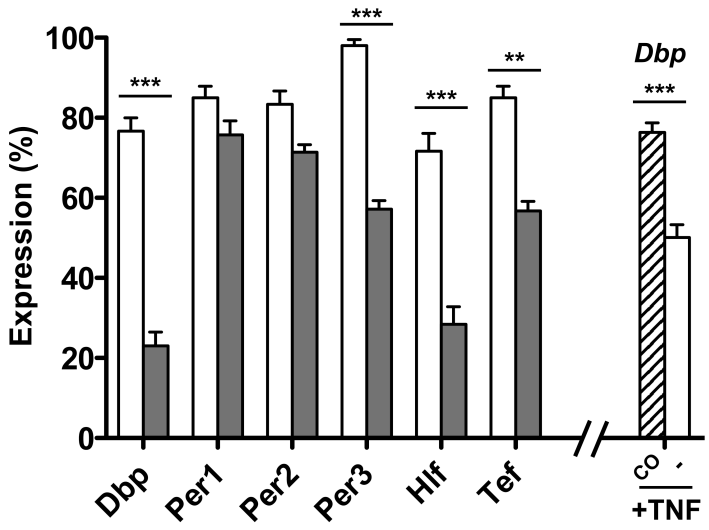


Figure 8: MEFs from WT mice (open bars) were compared to *Cirbp*$^{-/-}$ MEFs (gray bars) for their responsiveness to TNF. Data show the TNF induced expression of the indicated genes as percentage from their expression in untreated MEFs. . In a separate experiment the extent of TNF induced

inhibition of *Dbp* was assessed in *Cirbp*$^{-/-}$ MEFs which were complemented after transfection with pCMV6::Cirbp (CO) and in mock transfected *Cirbp*$^{-/-}$ MEFs (-). ** $p < 0.01$; *** $p < 0.001$

Cytokine effects on the expression of Dbp in Cirbp overexpressing cells.

To disclose *Cirbp* independent effects in selected clones in *Cirbp*$^{-/-}$ MEFs, the cells were transfected with a pCMV6::Cirbp expression vector. Overexpression of *Cirbp* in *Cirbp*$^{-/-}$ MEFs was found to render the cells less responsive to the inhibitory effect of TNF on *Dbp* expression (**Figure 8**). Next we studied the effect of overexpression of *Cirbp* in WT MEFs. Whereas WT MEFs treated with TNF (**Figure 9A and 9B**) or TGFβ (**Figure 9C and 9D**) showed a reduction of the expression of *Cirbp* and *Dbp* of around 50% compared to the untreated cells, overexpression of *Cirbp* led not only to a higher expression of *Dbp* but also made the cells unresponsive to the effect of the two cytokines to inhibit the expression of *Dbp* (**Figure 9A-D**).

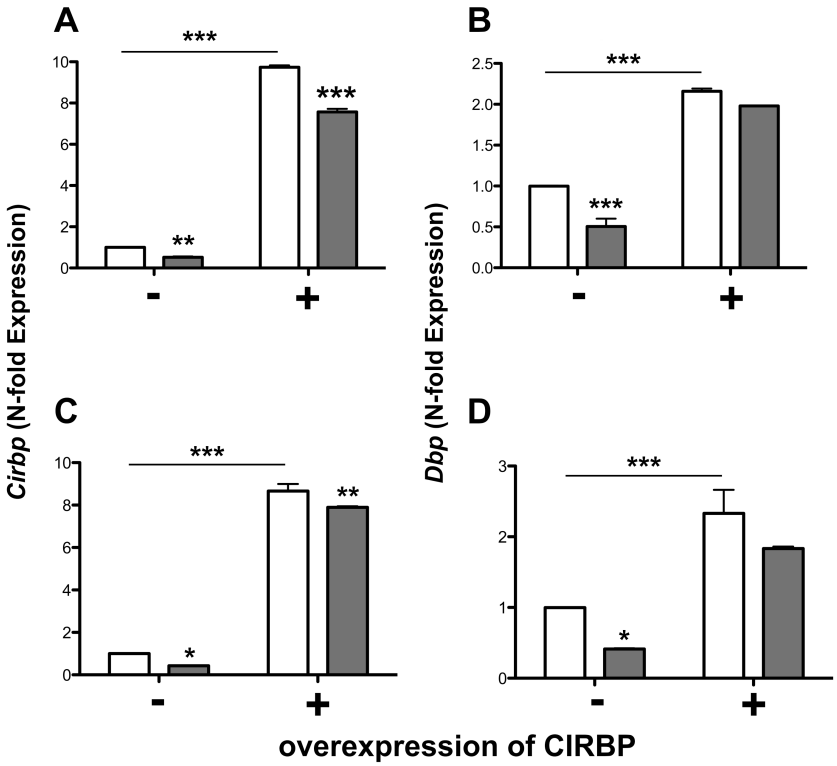


Figure 9: *Cirbp* overexpression in MEF cells treated with TNF (A and B) and TGFβ (C and D). WT MEFs (-) and *Cirbp* overexpressing MEFs (+) were treated with cytokines (grey bars) or medium control (open bars); the expression of *Cirbp* (A) and of *Dbp* (B) was assayed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Cirbp depleted cells show an augmented cytokine response to TNF treatment. Next we addressed the influence of TNF induced inhibition of *Cirbp* production on genes, which are not part of the molecular clock. Since some cytokines including TNF, IL-1β, CCL2 and CXCL12 as well as cytokine receptors, namely CXCR4 and CX3CR1, have been reported to display circadian rhythms (Scheiermann et al., 2013), we investigated the influence of *Cirbp* depletion on the TNF induced modulation of cytokine and cytokine receptor genes. Using real-time PCR we analyzed the expression of 94 genes in WT and *Cirbp*$^{-/-}$ MEFs. We find the TNF response in *Cirbp*$^{-/-}$ MEFs to be augmented in 73 (77%) of the 94 genes tested (supplementary Table 1). A reduced response was seen in 14 (15%) of the 94 genes (supplementary Table 2). The depletion of *Cirbp* was found to alter the expression of 5 of the 6 genes which have been described to display a rhythmic expression (see above), Compared to WT MEFs, *Cirbp*-/- MEFs treated with TNF showed an increase of *Cxcl12* (49.18-fold), *Cxcr4* (11.88-fold), *Cx3cr1* (7.41-fold) and *Il-1β* (2.48-fold) (**Figure 10**). While *Ccl2* expression was not different, the induction of *Tnf* by TNF treatment was inhibited (4.11-fold). These data indicate that *Cirbp* is involved in the modulation of TNF induced cytokine / cytokine receptor expression

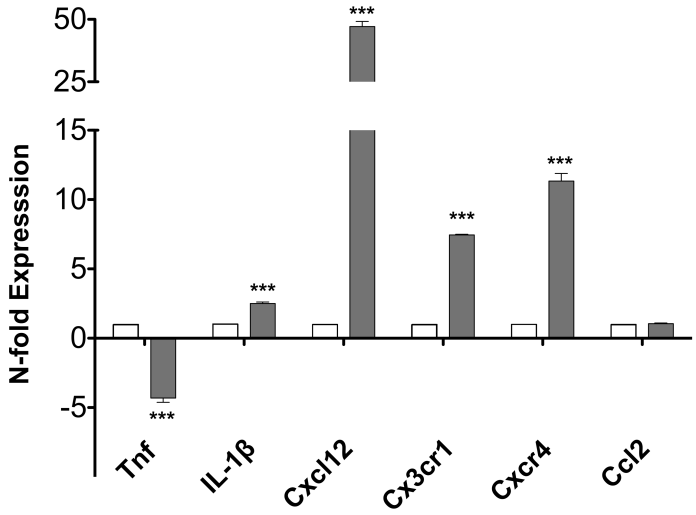


Figure 10: The effect of TNF on the expression of cytokines and cytokine receptors was tested on *Cirbp* depleted cells (grey bars) and WT MEFs (open bars). The expression of cytokine and cytokine receptor genes was set as 1-fold expression. *** $p < 0.001$

DISCUSSION

The data presented here show for the first time that TNF and TGFβ modulate clock gene expression by impairing the production of CIRBP. This conclusion is supported by the finding that overexpression of *Cirbp* protects cells from the inhibitory effects of TNF and TGFβ on the expression of clock genes. This contrasts the findings in *Cirbp* depleted cells, which show an increased susceptibility to the inhibitory effect of TNF on the expression of *Per3* and PAR-bZip clock genes including *Dbp, Hlf* and *Tef*. Since CIRBP is a RNA binding protein it may play multiple roles in gene expression by regulating the processing and fate of RNA transcripts. The stability of CIRBP target mRNAs has been shown to be increased in testis (Xia et al., 2012). CIRBP represses the usage of proximal polyadenylation sites (PAS) by binding to the common 3`-UTRs (Liu et al., 2012). It has been proposed that down regulation of CIRBP leads to the preferred use of the proximal PAS and shortening of 3-UTR (Liu et al., 2012). This effect would increase mRNA stability through reduction of microRNA mediated repression (Di Giammartino et al., 2011). When assessing the effect of CIRBP depletion we find the decline of *Dbp* mRNA over time not to be different in *Cirbp*$^{-/-}$ MEFs or TNF treated NIH-3T3 cells compared to respective controls. Thus, the effect of TNF to impair CIRBP production and thereby to modulate clock gene expression may involve other mechanisms. CIRBP has been shown to bind to *Clock* transcripts and to increase their cytoplasmic accumulation (Morf et al., 2012). The ectopic expression of *Clock* mRNA regulates high amplitude expression of genes, which show circadian oscillations including clock genes (Morf et al., 2012). Besides of its effect on *Cirbp* expression, TNF has been shown to interfere with E-box mediated transcription induced by CLOCK:BMAL1 (Cavadini et al., 2007). Transient transfections of NIH-3T3 cells with luciferase reporter genes and the native 3- or 1.7-kB promoter sequences of mouse *Per1* and *Per3* respectively, showed the promoter activity to be suppressed by TNF. Moreover, TNF inhibited the expression of E-box reporter constructs of the *Dbp* gene that was cotransfected with plasmids expressing CLOCK and BMAL1 proteins; the expression of mutated E-box reporter constructs was not altered by TNF (Cavadini et al., 2007). Studies using fibroblasts with a deletion of either *Per1* and *Per2* or *Cry1* and *Cry2* ruled out the possibility that TNF activates the repressor loop mediated by PER-CRY complexes (Petrzilka et al., 2009).

TNF and TGFβ effects on fibroblasts are in the center of cytokine-mediated pathologies in various immune-related diseases including rheumatoid arthritis, inflammatory bowel diseases and pulmonary fibrosis. By their effects on *Cirbp* expression, TNF and TGFβ may not only regulate expression of clock genes, but may also modulate immune-mediated effector functions. *Cirbp* protects MEFs from TNF induced apoptosis and neurons from H_2O_2 mediated cell damage (Li et al., 2012b; Sakurai et al., 2006). By lowering *Cirbp* expression, TNF may counteract *Cirbp* mediated anti-apoptotic effects. As outlined above, the expression of cytokines is influenced by the circadian clock (Scheiermann et al., 2013). When using cells deficient in *Cirbp* we find the TNF response to differ significantly. 73 (77%) of 94 cytokine / cytokine receptor genes showed an increased expression. Thus, *Cirbp* does not only play a role in the TNF induced dysregulated expression of clock genes, but may also influence the cytokine response in innate immunity and in immune mediated diseases.

The effect of TNF and TGFβ on CIRBP production and clock gene expression may play a pivotal role in the 24hr-based oscillations of physiological systems. Numerous studies show the circadian clock to participate in the regulation of metabolism (Bass and Takahashi, 2010; Turek et al., 2005). Oscillation of clocks in hepatocytes, which are entrained by feeding cycles, modulates gluconeogenesis through interference with glucagon and inhibition of cyclic AMP signaling (Zhang et al., 2010). As outlined in the introduction, glucose homeostasis is also influenced by clock genes through their action on insulin synthesis and sensitivity (Lamia and Evans, 2010; Marcheva et al., 2010; Schmutz et al., 2012). By modulating the expression of Nocturnin, clock genes regulate lipid absorption in the small intestine and adipogenesis (Stubblefield et al., 2012). *Clock* gene expression has been shown to be coupled to the sleep-wake distribution (Franken and Dijk, 2009). Several examples point to the existence of a crosstalk between cytokines and metabolism (Glass and Olefsky, 2012), TNF influences metabolic processes including glucose homeostasis, lipid metabolism and xenobiotic metabolism (Dantzer et al., 2008; Dinarello, 1987). Furthermore, TNF leads to changes of sleep-wake behavior and body temperature (Krueger, 2008; Morrow and Opp, 2004). TGFβ modulates key aspects of metabolic processes such as hepatic phospholipid and bile homeostasis. Both the organic solute transporter OSTβ and CYP7A1, the rate-limiting enzyme of bile acid synthesis, are regulated by TGFβ (Matsubara et al., 2012).

Moreover, TGFβ is involved in energy metabolism and energy-sensing pathways (Casalena et al., 2012; Yamada et al., 2011). The cytokine is induced by an increase of blood lactate and stimulates production of reactive oxygen intermediates by reducing complex IV and mitochondrial respiration. TGFβ enhances fatty acid oxidation and lipoprotein lipase (Yamazaki et al., 2002).

As outlined above, the circadian clock guides the daily oscillations of metabolism, sleep-wake behavior, endocrine pathways and of the immune response. TNF and TGFβ influence these physiological systems and interfere with the expression of the components of the hierarchical, multilayered regulatory network of the clock network (Cavadini et al., 2007; Gast et al., 2012; Kon et al., 2008). The data presented here, propose a new molecular circuit, which couples cytokine production with low levels of expression of clock genes. The linker is provided by CIRBP, which is required for efficient expression of *Clock* mRNA, but down regulated by TNF and TGFβ.

Author contributions

M.L., D.M., and A.M. designed and performed the experiments. P.F., J.F., and A.F. contributed to the concept of the study.

Conflict of Interest Statement

All authors declare that there are no conflicts of interest.

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SUPPLEMENTARY INFORMATION

Supplementary Table 1: Upregulated cytokine and cytokine receptor genes in TNF treated *Cirbp*$^{-/-}$ mouse embryonic fibroblasts

Gene	Gene symbol	Fold change
Chemokines		
Chemokine (C-X3-C motif) ligand 1	*Cx3cl1*	*393.44*
Chemokine (C-C motif) ligand 1	*Ccl1*	*134.36*
Chemokine (C-C motif) ligand 12	*Ccl12*	*129.79*
Chemokine (C-X-C motif) ligand 5	*Cxcl5*	*166.57*
Chemokine (C-C motif) ligand4	*Ccl4*	*117.78*
Chemokine (C-C motif) ligand 5	*Ccl5*	*49.52*
Chemokine (C-X-C motif) ligand 12	*Cxcl12*	*49.18*
Chemokine (C-X-C motif) ligand 9	*Cxcl9*	*33.13*
Chemokine (C-C motif) ligand 7	*Ccl7*	*17.88*
Chemokine (C-C motif) ligand 13	*Ccl3*	*16.45*
Chemokine (C-X-C motif) ligand 1	*Cxcl1*	*16.22*
Chemokine(C-X-C motif) ligand 10	*Cxcl10*	*9.51*
Chemokine receptors		
Chemokine (C-C motif) receptor 1	*Ccr1*	*106.15*
Chemokine (C-X-C motif) receptor 4	*Cxcr4*	*11.88*
Chemokine (C-X-C motif) receptor 2	*Cxcr2*	*11.71*
Chemokine (C-X-C motif) receptor 1	*Cxcr1*	*9.25*
Chemokine (C-X3C motif) receptor 1	*Cx3Cr1*	*7.41*
Chemokine (C-C motif) receptor 4	*Ccr4*	*5.70*
Chemokine (C-X-C motif) receptor 5	*Cxcr5*	*3.39*
Chemokine (C-C motif) receptor 8	*Ccr8*	*3.89*
Chemokine (C-C motif) receptor 3	*Ccr3*	*2.27*
Cytokines		
Interleukin 21	*Il-21*	*92.41*
Macrophage colony stimulating factor 1	*Csf1*	*64.89*
Interleukin-7	*Il-7*	*56.88*
Interleukin-15	*Il-15*	*37.79*
Interleukin-13	*Il-13*	*30.70*
Interleukin-17f	*Il-17f*	*24.94*
Leukemia inhibitory factor	*Lif*	*20.53*
Interleukin-1 alpha	*Il-1a*	*18.90*
Interleukin-3	*Il-3*	*17.39*
Interleukin-6	*Il-6*	*11.96*
Interleukine-18	*Il-18*	*10.01*
Interferon beta1	*Ifnb1*	*7.26*
Interleukine-12 alpha	*Il-12a*	*7.11*
Interferon gamma	*Ifng*	*6.36*
Interleukin-27	*Il-27*	*6.23*
Interleukin-1 receptor antagonist	*Il-1ra*	*4.92*
Interferon alpha1	*Ifna1*	*4.79*
Interleukin-4	*Il-4*	*3.30*
Interleukin 2	*Il-2*	*3.66*
Interleukin-1beta	*Il-1b*	*2.48*

Interleukin-15	*Il-5*	*2.39*
Interleukin-25	*Il-25*	*2.31*
Interleukin-17 alpha	*Il-17a*	*2.19*
Interleukin-11	*Il-11*	*2.08*
Interleukin-10	*Il-10*	*2.01*
Cytokine receptors		
Interleukin-1 receptor 1	*IlR1*	*225.97*
Interleukin-13 receptor alpha 2	*Il-13ra2*	*92.41*
Interleukin-1 receptor-like 1	*Il1Rl1*	*45.57*
Interleukin-6 receptor alpha	*Il6ra*	*11.88*
Interleukin 6 signal transducer	*Il-6st*	*10.56*
Interleukin 5 receptor alpha	*Il-5ra*	*7.46*
Interleukin-9 receptor	*Il-9r*	*5.78*
Interleukin-7 receptor	*Il-7R*	*5.54*
Leukemia inhibitory factor receptor	*Lifr*	*5.10*
Interleukin-1 receptor alpha1	*Il-11ra1*	*3.68*
Interleukin-22 receptor 1	*Il-22ra1*	*2.53*
Interleukin-27 receptor alpha	*Il-27Ra*	*2.25*
Interleukin-2 receptor alpha	*Il-2Ra*	*2.13*

Supplementary Table 2: Downregulated cytokine and cytokine receptor genes in TNF treated *Cirbp*$^{-/-}$ mouse embryonic fibroblasts

Gene	Gene symbol	Fold change
Chemokines		
Chemokine (C-C motif) ligand 28	Ccl28	-3.46
Chemokine receptors		
Chemokine (C-X-C motif) receptor 3	Cxcr3	– 25.11
Duffy antigen receptor for chemokines	Darc	-17.15
Chemokine (C-X-C motif) receptor	Ccr5	-3.68
Cytokines		
Interleukin-23 alpha	Il-23a	-7.62
Tumor necrosis factor	Tnf	-4.11
Interleukin-28 beta	Il28b	-3.97
Interleukin-24	Il-24	-3.84
Cytokine receptors		
Interleukin-2 receptor beta	Il2rb	-57.68
Interleukin-10 receptor beta	Il10rb	-37.79
Interleukin-12 receptor beta2	Il12rb2	-7.26
Interleukin-17 receptor alpha	Il-17ra	-2.22
Interleukin-21 receptor	Il-21r	- 2.28

The cytokine and cytokine receptor genes shown are those that meet the significance cutoff set at -2.0-fold change and p-values <o.ooo1

3.4 Transforming growth factor-beta inhibits the expression of clock genes

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ABSTRACT

Disturbances of sleep–wake rhythms are an important problem in Alzheimer's disease (AD). Circadian rhythms are regulated by clock genes. Transforming growth factor-beta (TGF-β) is overexpressed in neurons in AD and is the only cytokine that is increased in cerebrospinal fluid (CSF). Our data show that TGF-β2 inhibits the expression of the clock genes *Period* (*Per*) *1*, *Per2,* and *Rev-erbα*, and of the clock-controlled genes D-site albumin promoter binding protein (*Dbp)* and thyrotroph embryonic factor (*Tef*). However, our results show that TGF-β2 does not alter the expression of brain and muscle Arnt-like protein-1 (*Bmal1*). The concentrations of TGF-β2 in the CSF of 2 of 16 AD patients and of 1 of 7 patients with mild cognitive impairment were in the dose range required to suppress the expression of clock genes. TGF-β2-induced dysregulation of clock genes may alter neuronal pathways, which may be causally related to abnormal sleep-wake rhythms in AD patients.

INTRODUCTION

The neuropathological hallmarks of Alzheimer's disease (AD) are neuronal loss, senile plaques, and neurofibrillary tangles in cortical and limbic regions. A major component of senile plaques is the β-amyloid (Aβ), which is cleaved by β- and γ-secretases from the amyloid precursor protein (APP). Generation and clearance of Aβ have been shown to be modulated by the immune system. Transforming growth factor-beta (TGF-β) 1 drives the expression of APP in astrocytes and enhances the generation of Aβ (Lesne et al., 2003). Overexpression of TGF-β1 in the brain promotes brain inflammation, paralleled by increased deposition of brain vascular Aβ and reduction of Aβ deposits in the parenchyma

However, inhibition of TGF-β signaling in peripheral macrophages has been found to lead to an influx of macrophages into the brain and enhancement of Aβ clearance (Town et al., 2008). TGF-β2 induces neuronal cell death by binding to the extracellular domain of APP (Hashimoto et al., 2005; 2006). The expression of TGF-β2 is increased in glial cells located near the senile plaques in transgenic mice that overexpress the Swedish type of familial AD (Hashimoto et al., 2006). Furthermore, TGF-β2 is elevated in neurons of the hippocampus and cortex of patients with AD (Noguchi et al., 2010). TGF-β-mediated gene transcription involves phosphorylation of Smad2 and Smad3, which thereby associate with Smad4 and translocate to the nucleus. In neurons of AD patients, phosphorylated Smad2 is overexpressed in the cytoplasm, but not in the nucleus of neurons located in neurofibrillary tangles and granulovascuolar degeneration (Lee et al., 2006). The expression of TGF-β and pSMAD3, the activated form of the TGF-β receptor-mediated transcriptional modulator, follows a circadian pattern (Beynon et al., 2009). A recent meta-analysis of cytokine expression in AD revealed that many proinflammatory cytokines, including IL-1β, IL-6, IL-12, IL-18, and TNF, are increased in the blood of patients. However, TGF-β is the only cytokine that is significantly elevated in patients' cerebrospinal fluid (CSF) (Swardfager et al., 2010). Besides TGF-β1, its functionally related isoform TGF-β2 is increased in the CSF as well (Vawter et al., 1996). However, the increase observed failed to reach significance.

In studies of mice that overexpressed TGF-β1 in the hippocampus, TGF-β1 was found to influence social interaction and depressive-related behavior (Depino et al., 2011). It has not been assessed whether TGF-β modulates behavior independent of its role in Aβ

production and clearance. Patients with AD show an impairment of memory that has been thought to result from alterations in the hippocampus, a critical brain area for memory processing (Morris, 2012; Nadel and Moscovitch, 1997; Squire and Alvarez, 1995). Recent studies suggest that memory decline in AD could result from impairment of sleep-dependent memory consolidation (Rauchs et al., 2005; 2008). In amnestic mild cognitive impairment, which precedes AD, inadequate memory consolidation is associated with impaired sleep (Westerberg et al., 2010). AD patients show disturbances of the circadian rhythm (for review, see Weldemichael and Grossberg, 2009). Daytime agitation, nighttime insomnia, and restlessness are among the common behavioral changes in AD.

It is hypothesized that the abnormal sleep-wake behavior in AD may be due to alterations of the molecular clock that maintains the circadian rhythm. The integration of the day and night variation is provided by the suprachiasmatic nucleus (SCN) of the hypothalamus, which receives light information from the retina and synchronizes the clock genes of other brain areas and peripheral organs. Every cell of the body contains functional feedback loops composed of the positive transcriptional activators *Clock* and *Bmal1* and negative elements, including *Per* and cryptochrome (*Cry)*. CLOCK and BMAL1 heterodimerize and bind to E-box enhancer sequences to promote transcription of the period genes *Per1* and *Per2,* and of the cryptochrome genes *Cry1* and *Cry2,* which themselves inhibit the activity of CLOCK-BMAL1. A second feedback loop, comprising REV-ERBα, controls the expression of the positive clock element *Bmal1* (Hastings, 2003; Schibler, 2009).

In this study, we assessed the role of TGF-β2 on clock gene expression in fibroblasts and neuronal cells. We found that in CSF of AD patients, detectable concentrations of TGF-β2 profoundly inhibited the expression of *Period* genes, *Rev-erbα,* and the clock-controlled genes *Dbp* and *Tef*.

METHODS

Participants

Patients with mild cognitive impairment (MCI) and AD were recruited from the Memory Clinic at the Division of Psychiatry Research and Psychogeriatric Medicine,

University of Zurich. Non-demented control subjects were recruited from the Departments of Neurology and Anesthesiology at the University Hospital Zurich. The study was approved by the local ethics committee. Written informed consent was obtained from all subjects before the investigation. Additional consent was obtained from caregivers in the case of dementia.

The cognitively impaired group (CI) was comprised of 7 (4 female) subjects with MCI (mean age 69.4 years ± 9.7, mean MMSE 26.7 ± 1.5) and 16 (8 female) subjects with AD (mean age 71.1 years ± 9.1, mean MMSE 17.8 ± 3.5). Patients with AD were diagnosed as "probable AD" according to the NINCDS-ADRDA criteria and "Alzheimer's dementia" according to ICD (F 00.0 and F 00.1) after extensive clinical work-up (medical history, clinical examination, ECG, MRI or CCT, blood and urine laboratory assessments) (McKhann et al., 1984).

Psychometric testing

The psychometric test battery followed the CERAD procedure, including verbal fluency, the Boston naming task, recall and delayed recall tests, and the Mini-Mental State Examination (MMSE). MCI subjects were diagnosed according to Petersen et al. (1999), requiring documented impairment in one memory test and independent daily living. The group of non-demented controls (N = 10, 5 females, mean age 65.1 ± 7.3) consisted of subjects without evidence for cognitive disturbances by clinical examination and judgment of the physician in charge, who underwent lumbar puncture at the Department of Anesthesiology (n = 7) or Neurology (n = 1) or at the memory clinic (n = 2).

Cerebrospinal fluid sampling and quantification of TGF-β1 and TGF-β2 in CSF

Spinal taps were done between lumbovertebral body 3 and 4 or 4 and 5 using iodine solution (Braunol, B. Braun Medical AG, 6204 Sempach, Switzerland) as disinfectant and Sprottecanula 21G × 31/2 (99 mm) for punctuation needles. The time point of punctuation was between 8 a.m. and 5 p.m. 6 ml of cerebrospinal fluid were collected in 13 ml polypropylene tubes (round bottom, screw caps high density polypropylene) and immediately sampled in approximately 24 sterile caps (0.5 ml, Screw Cap Tubes, Art. Nr. SCT-050-C-S, Axygen Scientific, Union City, CA) with 250 µl volume each.

TGF-β was measured using the Quantikine human TGF-β1 and TGF-β2 immunoassay from R&D (Abingdon, UK). To activate latent TGF-β1 to the immunoreactive form, acid activation and neutralization was performed according to the recommendations from R&D.

Clock gene expression in TGF-β2–treated cells

Murine fibroblast cells, NIH 3T3 (CRL-1658), were obtained from the American Type Culture Collection. To generate mouse embryonic fibroblasts (MEF), embryos of *Smad3* homozygous wild type (WT) or knockout (KO) from the same breeding were used at day 12 of gestation (E12). Liver and head were removed and the remaining tissue was digested with 0.25% trypsin-EDTA in Dulbecco's modified eagle medium (DMEM) for 30 min at room temperature. The dissociated cells were plated in DMEM containing 20% fetal calf serum (FCS). They were split 1:4 until they reached passage 10. Cells were used within passage 10–30. NIH 3T3 fibroblasts and MEFs were grown in DMEM (Gibco, Basel, Switzerland), supplemented with 10% phosphate-buffered saline (PBS) (PAA Laboratories, Pasching, Austria) and Glutamax (Gibco). For TGF-β2 treatment, cells were grown to confluency, and then the medium was replaced by serum- free DMEM/Glutamax with or without TGF-β2 (recombinant human TGF-β2; mammalian derived; 100–35B) from Peprotech (London, UK). After various time points of cell treatment, tissue culture plates were washed once with the ice-cold PBS solution and kept at -70 °C until the extraction of whole-cell RNA.

The mouse hippocampus neuronal cell line HT22 was obtained from David Schubert at the Salk Institute (La Jolla, CA). HT22 cells were plated in 12-well tissue culture plates (100,000 cells per well) in DMEM with 10% FCS. Two days after plating, cultures were serum deprived for 1 hour. Thereafter, HT22 cells were treated with TGF-β2 for 4 hours.

Whole cell RNA from cultured cells was extracted using TRIzol (Invitrogen; Life Technologies Europe, Zug, Switzerland) or peqGOLD RNAPure (peqLab) according to the manufacturer's instructions. Subsequently, RNA was reverse-transcribed using random hexamers (Roche) and M-MuLV reverse transcriptase (Applied Biosystems). The cDNA equivalent to 20 ng of total RNA was PCR amplified in an ABI PRISM HT7900 detection system (PE- Applied Biosystems) using the TaqMan Universal PCR

Master Mix (Applied Biosystems) and quantified as follows. Primers and probes for Taqman analysis were either purchased from Applied Biosystems or purchased from Microsynth (Balgach, Switzerland). With the exception of the *Runx3* and *Dec1* primers, all other primers have been described previously (Petrzilka et al., 2009). The primers used for *Runx3* were 5-ACCGCTTTGGAGACCTGCGCATG-3 and 5-CGCTGTAGGGGAAGGCGGCAGA-3, and for *Dec1*, 5-GAGACCCTGCGATCCTCCC-3 and 5- AGGTCTCCGTGCTCCAGCC-3. The relative levels of each RNA were calculated by the $2^{-\Delta\Delta Ct}$ method (Ct standing for the cycle number at which the signal reaches the threshold of detection); *Gapdh* mRNA was used as a housekeeping gene. Each Ct value used for these calculations is the mean of two duplicates of the same reaction. Relative RNA levels are expressed as *x*-fold variations compared with untreated.

Statistics

Statistical analyses were calculated using Student's *t*-test (GraphPad Prism version 4.0).

RESULTS

TGF-β2 impairs the expression of clock genes

Previous reports indicate that *in vitro* cultures of fibroblasts provide an excellent tool to describe circadian expression of central clock and clock- controlled genes for at least three cycles (Nagoshi et al., 2004). For the study presented here, 3T3 fibroblasts were exposed to various doses of TGF-β2. This cytokine, rather than its isoform TGF-β2, was chosen because TGF-β2 is present in the CSF in higher concentrations than TGF-β1 (see below). However, both TGF-β1 and TGF-β2 bind to the same receptor TGF-βRII and share their biological activities. The expression of the clock-controlled gene *Dbp* was assessed after 24 hours by RT-PCR. TGF-β2 profoundly inhibited *Dbp* expression. A total of 75% inhibition of *Dbp* expression was seen with 100 pg/ml TGF-β2, and complete suppression of *Dbp* expression was achieved with TGF-β2 at 5 ng/ml (Fig. 1A). A statistically significant effect of TGF-β2 required its presence for 4 h in fibroblast cultures (data not shown). Next, we determined whether TGF-β2 alters the rhythmic

expression of clock genes. Cultures were treated with TGF-β2 and thereafter RNA was extracted every 4 h. TGF-β2 did not alter the phasic expression of *Dbp*, but rather suppressed its maximum oscillation (Fig. 1B). To gain a more complete picture of the role of TGF-β2 in clock gene expression, the effect of the cytokine was also tested on *Per1*, *Per3*, *Tef*, *Rev-erbα* and the central core clock genes *Clock* and *Bmal1*. Whereas TGF-β2 did not influence the expression of *Bmal1* and had only a minor effect on the expression of *Clock* (20% inhibition), the other clock genes analyzed were inhibited by >70% when compared with untreated cultures (Fig. 1C). TGF-β2 also inhibited the expression of *Dbp* in HT22 neuronal cells. This effect was dose dependent and paralleled by an increase of *Dec1* (Fig. 1D). When TGF-β2 was added in a concentration of 2 ng/ml, the suppression of *Dbp* was 49% and the induction of *Dec1* was 3.7-fold.

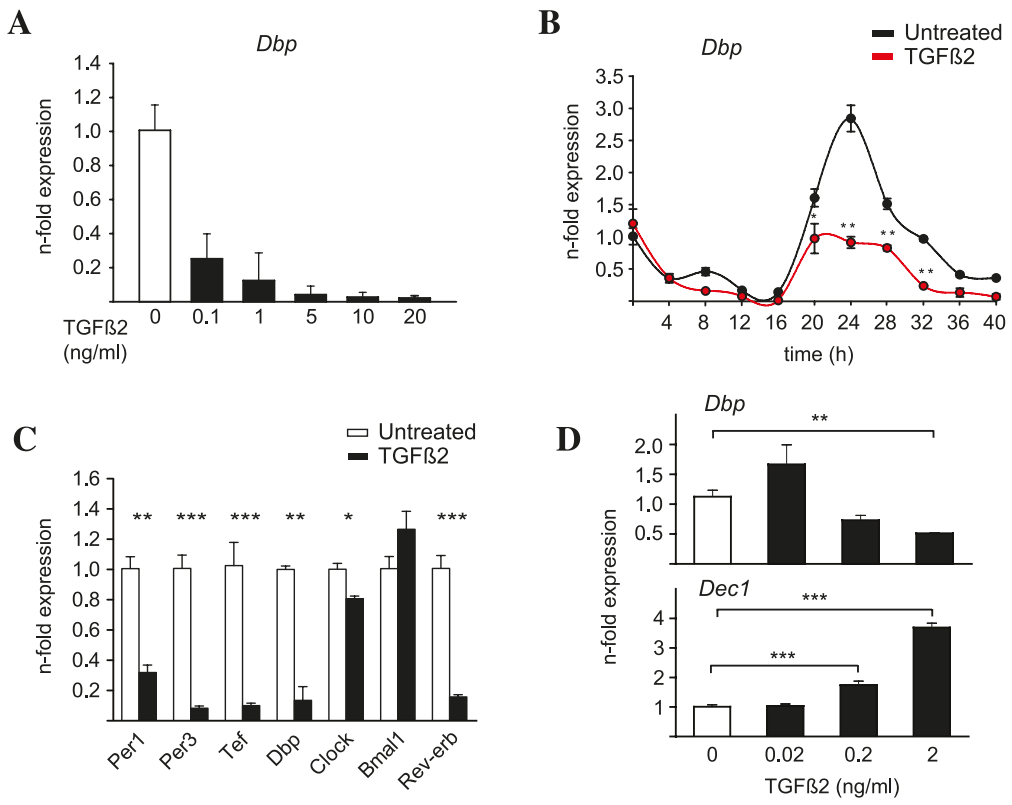

Figure 1: TGF-β2 suppresses the expression of clock genes in NIH3T3 fibroblasts and HT22 neurons. (A) Dose-dependent downregulation of Dbp gene expression. (B) TGF-β2 (1 ng/ml) reduces the amplitude of Dbp expression in synchronized NIH3T3 fibroblasts. (C) TGF-β2 (1 ng/ml) -induced inhibition of Per1, Per3, Tef, Dbp, Rev-Erbα, and Clock, but not of Bmal1. (D) TGF-β2 inhibits the expression of Dbp in HT22 neuronal cells, the effect being paralleled by an upregulation of Dec1. All experiments were done in triplicate cultures. Data show the mean ± SEM. *** $p < 0.0005$; ** $p < 0.005$; * $p < 0.05$.

Smad3 signaling is only partially involved in the suppression of Per3 and Dbp by TGF-β2

Binding of TGF-β to TGF-βRII leads to phosphorylation of TGF-βRI. This step is followed by recruitment and phosphorylation of Smad2 and Smad3 that act together as heterodimer. In further experiments, we tested TGF-β2 on MEFs, which were established from *Smad3* gene knockout mice (Smad3$^{-/-}$). To verify SMAD3 deficiency at a functional level, the response of Smad3$^{-/-}$ MEFs to TGF-β2 was assessed by investigating the expression of *Runx3*, a well-established TGF-β target gene (Ito and Miyazono, 2003). TGF-β2 failed to induce *Runx3* in Smad3$^{-/-}$MEFs, but led to a profound increase in WT cells (Fig. 2A). Upon treatment of Smad3$^{-/-}$ MEFs with TGF-β2, the induced suppression of both *Dbp* and *Per3* expression was significantly less pronounced compared with WT MEFs (Fig. 2B and C). The percentage of TGF-β2–induced inhibition of expression of *Dbp* in wild-type and Smad3$^{-/-}$ MEFs was 85.8 ± 2.5% and 46.9 ± 8.1% ($p < 0.0001$), respectively. Likewise, the TGF-β2–induced inhibition of expression of *Per3* was more pronounced in WT compared with Smad3$^{-/-}$ MEFs, the respective values being 63.8 ± 10.4% and 25.3 ± 7.3% ($p < 0.003$). However, the data show a significant Smad3-independent inhibitory effect of TGF-β2 on the expression of *Dbp* and *Per3* (Figs. 2B and C). The same results were seen when testing another eight MEF cell clones established from Smad3$^{-/-}$ mice (data not shown). A recent study showed TGF-β induces the basic helix-loop-helix protein *Dec1,* an effect that is Smad3-dependent (Kon et al., 2008; Zawel et al., 2002). *Dec1* has been described to be involved in the expression of clock genes (Honma et al., 2002). Our data confirm that TGF-β upregulates *Dec1* expression in WT MEFs, but not in Smad3$^{-/-}$ MEFs (Fig. 2D).

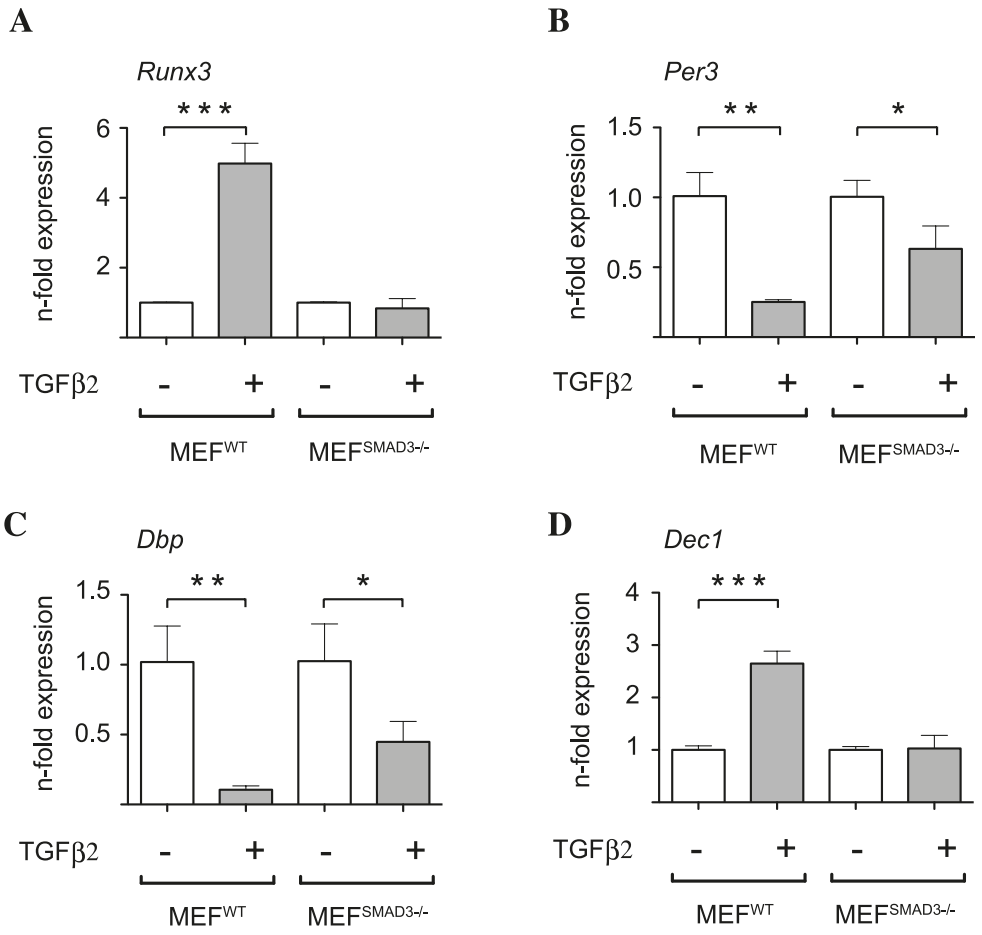

Figure 2: *The suppression of Dbp and Per3 by TGF-β2 is not exclusively regulated by Smad3.* (A and D). The upregulation of Runx3 and Dec1 by TGF-β2 is completely abolished in Smad3-deficient MEFs. (B and C) Downregulation of Per3 and Dbp is only partly dependent on Smad3. All experiments were done in triplicate cultures. Data show the mean ± SEM. ***P <0.0005; **P <0.005; *P <0.05.

TGF-β1 and TGF-β2 concentrations in the CSF of AD patients

On the basis of the intriguing function of TGF-β2 to modulate the expression of clock genes, we elected to study CSF samples of patients with MCI (n = 7) and AD (n = 16), the CSF of both patient groups hereby named CSF-CI (cognitively impaired (CI)). The reported concentration of TGF-β in CSF-CI varies significantly, ranging from between 0.2 ng/ml and 30 ng/ml (Rota et al., 2006; Tarkowski, 2002). The reasons for the variability are not entirely clear, but may be due to TGF-β being present in latent form. Activation of the latent form can be achieved by heat or acid treatment. To overcome variabilities due to CSF storage and freeze-thaw cycles, we acidified the CSF prior to testing in a commercial ELISA system. As shown in Figure 3, TGF-β1 can be detected in the CSF-CI and in healthy controls (HC). The mean ± SD for TGF-β1 in CSF-CI was

higher compared with HC, the concentrations being 51.09 ± 13.08 pg/ml and 36.16 ± 28.50 pg/ml, respectively. Since TGF-β1 and its isoform TGF-β2 are increased in the brain of AD patients, we assessed TGF-β2 in our ELISA system as well. In CSF-CI, the mean concentrations of TGF-β2 were higher compared with HC, the mean ± SD for CSF-CI and HC being 50.09 ± 28.43 and 36.36 ± 27.31, respectively. The difference found did not reach statistical significance. When dissecting the CSF-CI into samples from AD and MCI patients, no differences in the concentration of TGF-β1 and TGF-β2 were found (data not shown). In two AD patients and one patient with MCI, the TGF-β2 concentrations were above 90 pg/ml, the mean ± 2 SD of HC. In these three CSF samples, the TGF-β2 CSF concentrations measured was between 95 pg/ml and 115 pg/ml.

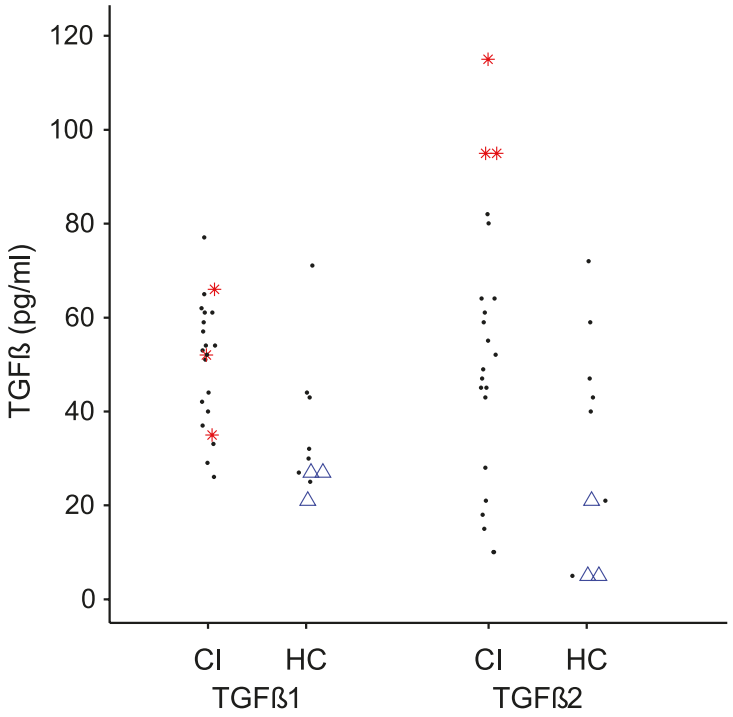

Figure 3: TGF-β1 and TGF-β2 in CSF of CI patients and HC. Stars indicate three CSF samples (two AD patients, one MCI patient) with highest concentrations of TGF-β2, and open triangles refer to the three CSF samples with the lowest TGF-β2 concentrations. For identification of these CSF samples, the same symbols were used in the data showing the concentrations of TGF-β.

CSF of CI patients neither suppresses the expression of clock genes nor induces the TGF-β target gene Dec1

Since in the three CSF samples outlined previously, the TGF-β2 concentrations measured (around 0.1 ng/ml) were in the range of those required for significant suppression of the *Dbp* gene (Figs. 1B and Fig. 3), we evaluated the impact of CSF-CI on the expression of clock genes. To overcome the problem of small volumes of the CSF samples used, we formed a pool with the three CSF samples with the highest TGF-β2 concentrations (see symbol * in Fig. 3), This pool failed to suppress the expression of *Dbp, Per1,* and *Per3* (data not shown). Since the CSF pools had to be diluted fourfold in the assay, the absence of effects of the CSF pool may be due to TGF-β2 concentrations that are under the detection limit. Alternatively, CSF samples may harbor factors that hinder TGF-β2 to act on the expression of clock genes. When adding TGF-β2 to CSF samples derived from CI patients with low TGF-β2, the CSF samples supplemented with the higher TGF-β2 concentration (2 ng/ml) were found to inhibit the expression of *Dbp* and to enhance the expression of *Dec1* (Fig. 4). The effect on *Dbp* expression did not become visible when the CSF samples were supplemented with less TGF-β2 (0.1 ng/ml). These data indicate that the absence of downregulation of clock genes by the CSF from patients with CI is not due to the presence of factors in the CSF, which would interfere with the biological function of TGF-β2 to modulate clock gene expression.

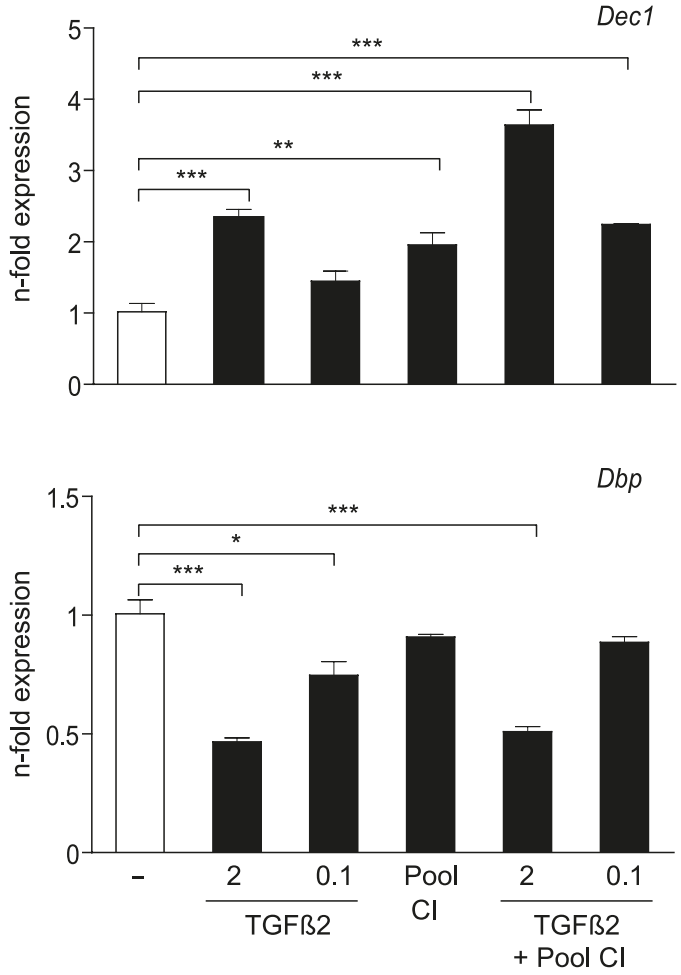

Figure 4: *CSF from patients with CI does not impair the effect of TGF-β2 on the expression of Dbp and Dec1.* HT22 neuronal cells were treated either with TGF-β2 (2 ng/ml or 0.1 ng/ml) or with a CSF pool that was derived from three CI patients with the lowest TGF-β2 concentrations (pool CI). Before testing, the pool was supplemented with TGF-β2 (2 ng/µl or 0.1 ng/ml). The experiments were done in triplicate cultures. Data show the mean ± SEM. ***P <0.0005; **P <0.005; *P <0.05.

DISCUSSION

In AD, sleep efficiency is reduced and sleep-wake rhythms disturbed (for review see Weldemichael and Grossberg, 2009). Disruption of sleep-wake rhythm is the most frequent reason for nursing home placement (Lebert et al., 1996). Interestingly, the rhythmic expression of the clock genes *Bmal1*, *Cry1,* and *Per1* was diminished in preclinical and clinical AD (Wu et al., 2006). In our study, we focused on the modulation of clock gene expression by TGF-β, because according to a recent meta-analysis on cytokines in AD, TGF-β1 is the only cytokine identified to be increased in

the CSF of AD patients. TGF-β1 and TGF-β2 are expressed on astrocytes and neurons in AD patients, and inhibition of the TGF-β signaling pathways in mice overexpressing Aβ, mitigates the disease (references are given in the introduction). Using fibroblasts and HT22 neuronal cells, we found that TGF-β2 suppressed the expression of *Per1*, *Per2,* and *Rev-erbα* and the clock- controlled genes *Dbp* and *Tef*. However, the expression of the *Bmal1* gene was not significantly altered. An analogous effect has been described in studies on TNF, which impairs binding of Clock-Bmal1 heterodimers to the E-box sequences of *Dbp* and *Rev-erbα* (Taraborrelli et al., 2011).

The mechanisms involved in the TGF-β2-induced inhibition of E-box-regulated clock genes are not yet clear. In Ras-1 fibroblasts, which stably express a luciferase reporter under the regulation of a 0.3-kb *Bmal1* promoter, TGF-β1 leads to a phase-shift of the circadian oscillations of the bioluminescence signal (Kon et al., 2008). This effect of TGF-β1, which is mediated by activation of the activin receptor-like kinase, was also associated with suppression of *Dbp* and *Rev-erbα* as well as an increased expression of the *Dec1. Dec1* (also known as Stra13), which is induced by TGF-β, is a basic helix-loop-helix protein that can act as a repressor of gene transcription (Zawel et al., 2002). Our data show that TGF-β2 not only inhibits *Dbp* and *Rev-erbα* in fibroblasts, but also leads to a prominent decrease of *Per1, Per3,* and *Tef* at the time point of an unaltered expression of *Bmal1*. Furthermore, our time-course analysis of *Dbp* expression revealed that TGF-β2 inhibits the amplitude of *Dbp* oscillation without causing a phase-shift of its expression. TGF-β-induced signaling involves the activation of Smad proteins. As reported by others, we find the induction of *Dec1* to be dependent on expression of Smad3 in TGF-β2 treated fibroblasts (Kon et al., 2008). However, despite the absence of *Dec1* expression in Smad3$^{-/-}$ MEFs, TGF-β2 was still able to suppress the expression of *Per3* and *Dbp* - the effect, however, being less pronounced. This is of note since TGF-β1 causes a phase-shift of the expression of *Dbp* and of *Per1 in vivo* in mice in a *Dec1*-dependent manner (Kon et al., 2008). Smad-independent effects of TGF-β1 occur through various pathways, including the Ras-extracellular signal-regulated kinase (Erk), TGF-β1-activated kinase 1 (TAK1), and phosphatidylinositol 3-kinase (PI3K)-Akt pathways (Eger et al., 2005).

In a meta-analysis of cytokines detected in AD, CSF TGF-β1 was found to be the only cytokine elevated (Swardfager et al., 2010). This contrasts with levels of TNF, IL-1, and

IL-6, which were not increased. The analysis comprised five studies with a total of 113 AD patients and 114 age-matched controls. All five studies investigated the CSF concentrations of TGF-β1, but not of TGF-β2. In addition, the amount of TGF-β1 in CSF correlated with the MMSE scores (Rota et al., 2006). TGF-β2 is synthesized in AD by hippocampal and cortical neuronal cells and upregulated in familial AD cases, with presenilin mutations in astrocytes around senile plaques and in neurons with neurofibrillary tangles (Noguchi et al., 2010). In homogenates of brain tissue of AD, TGF-β is 3.2 times the average level of control samples(Noguchi et al., 2010). TGF-β2 has only been assessed in the CSF in one study, where it was increased compared with controls, but the effect was not statistically significant (Vawter et al., 1996). Overexpression of TGF-β in neurons and plaques of AD patients may not be mirrored, on a quantitative level, in corresponding changes in the CSF TGF-β content. The comparison between CNS tissue and CSF is complex because binding of TGF-β to TGF-βRII in brain tissue may lead to an underestimation of TGF-β in the CSF. Our data show that 2 out of 16 AD patients and 1 out of 7 patients with MCI had TGF-β2 CSF concentrations above 90 pg/ml. This is remarkable because a TGF-β2 concentration of 100 pg/ml led to a 75% inhibition of *Dbp* expression. However, a pool of the three CSF samples with the highest TGF-β2 levels did not mimic the effect on clock genes as seen with recombinant TGF-β2. When supplementing CSF samples with TGF-β2, the effect of the cytokine to inhibit the expression of *Dbp* and to enhance the expression of *Dec1* in HT22 neuronal cells becomes detectable. These experiments indicate that there are no factors in the CSF that would override the activity of TGF-β2 on clock gene expression, and the dilutions of the CSF samples required in the clock gene assay are too high to allow for the functional detection of TGF-β in the CSF.

Here, we document the efficacy of TGF-β2 to downregulate central clock genes (*Period* genes) and clock-controlled genes (*Dbp, Tef*), but not the master clock gene *Bmal1*. The significance of these findings is provided by the observation that patients with AD have disturbances of the circadian rhythm, and the concept that the timing of sleep and wakefulness and sleep structures results from the interaction of a circadian and a sleep-wake-dependent homeostatic process (Borbély, 1982; Weldemichael and Grossberg, 2009). Findings in gene KO mice with inactivation of individual clock genes led to the hypothesis that circadian clock genes, in addition to controlling circadian timing of

sleep, may also be important to sleep-regulatory processes (Franken et al., 2001). For example, while total sleep time remains constant in *Per1* and *Per2* mutant mice, the distribution of sleep is affected by the mutation. Collectively, the data presented indicate that TGF-β-induced dysregulation of clock gene expression may play a role in abnormal sleep-wake rhythms in Alzheimer's disease.

Author contributions

H.G., S.G., S.P., M.L, and A.M. designed and performed the experiments. A.G., C.H., T.B., and A.F. contributed to the concept of the study.

Acknowledgments

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Conflicts of interest

The authors declare no conflicts of interest.

3.5 Cytokine-induced sleep: Neurons respond to TNF with production of chemokines and increased expression of Homer1a in vitro

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ABSTRACT

Interactions of neurons with microglia may play a dominant role in sleep regulation. TNF may exert its somnogenic effects by promoting attraction of microglia and their processes to the vicinity of dendrites and synapses. We found TNF to stimulate neurons (i) to produce CCL2, CCL7 and CXCL10, chemokines acting on mononuclear phagocytes and (ii) to stimulate the expression of the macrophage colony stimulating factor (M-CSF/Csf1), which leads to elongation of microglia processes. TNF may also act on neurons by affecting the expression of genes essential in sleep–wake behavior. The neuronal expression of Homer1a mRNA increases during spontaneous and enforced periods of wakefulness. Mice with a deletion of Homer1a show reduced wakefulness with increased non-rapid eye movement (NREM) sleep during the dark period. Recently, the TNF-dependent increase of NREM sleep in the dark period of mice with CD40-induced immune activation was found to be associated with decreased expression of Homer1a. In the present study we investigated the effects of TNF and IL-1β on gene expression in cultures of the neuronal cell line HT22 and cortical neurons. TNF slightly increased the expression of Homer1a and IL-1β profoundly enhanced the expression of Early growth response 2 (Egr2). The data presented here indicate that the decreased expression of Homer1a, which was found in the dark period of mice with CD40-induced increase of NREM sleep is not due to inhibitory effects of TNF and IL-1β on the expression of Homer1a in neurons.

INTRODUCTION

Patients with autoimmune diseases suffer from sickness behavior syndrome (SBS), which is characterized by fatigue, malaise, decreased appetite, weight loss, and reduced social activities (Dantzer et al., 2008). A causal link between tumor necrosis factor (TNF) and SBS is suggested because treatment with the soluble TNF receptor p75 or with antibodies against TNF improved fatigue and depression in patients with rheumatoid arthritis (RA), psoriasis, and Crohn's disease (Farahani et al., 2006; Katz et al., 2009; Lichtenstein et al., 2002; Moreland et al., 2006; Taylor and Feldmann, 2009; Tyring et al., 2006). In recent studies on experimental SBS in mice, the immune activation was triggered by anti-CD40 monoclonal antibodies (mAb), which activate the CD40 receptor in B cells and antigen presenting cells including macrophages and dendritic cells. Mice treated with CD40 mAb show a decrease in wakefulness and an increase in non-rapid eye movement (NREM) sleep during the dark period (Gast et al., 2013; Taraborrelli et al., 2011). Inactivation of the TNF receptor 1 gene or treatment with soluble TNF receptor p75 protects mice from CD40 mediated sleep–wake changes, but not from immune activation (Gast et al., 2013; Nimmerjahn et al., 2005; Taraborrelli et al., 2011).

Reciprocal signaling between neurons and microglia may be essential in remodeling of brain circuits including the formation, modification, and elimination of synaptic structures. This concept is supported by evidence that microglial processes periodically contact dendritic spines and axon terminals in vivo (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009). Contacts between neurons and microglia foster cell–cell communication via membrane receptors and soluble mediators including cytokines. Among the latter, TNF is considered to play an important role in immune-mediated behavior changes and inflammation. In inflammatory diseases, TNF is produced mainly by microglia and macrophages. Examples are experimental autoimmune encephalomyelitis and murine cerebral malaria and (Medana et al., 1997; Renno et al., 1995). As shown by in situ hybridization the type of cell producing TNF in rats injected intravenously with lipopolysaccharide (LPS) was identified as microglia, but not as neurons and astrocytes (Buttini et al., 1997). Using in situ hybridization and immunohistochemistry microglia and macrophages were characterized as the major sources of TNF in middle cerebral artery occlusion in mice

(Gregersen et al., 2000). However, immunohistochemical studies show that the physiological role of TNF in sleep regulation may be mediated by TNF production in neurons (Churchill et al., 2008). TNF binds to TNF receptor TNFR1 and TNFR2, both of which are expressed on neurons (Marchetti et al., 2004). The production of TNF is strongly influenced by circadian rhythms. Levels of Tnf mRNA and TNF correlate with sleep propensity, that is, high sleep propensity is associated with high levels of TNF mRNA and protein. Furthermore increased TNF is seen during sleep deprivation (Imeri and Opp, 2009; Kaushal et al., 2012; Krueger, 2008). Central or systemic injections of TNF or IL-1β increase the duration of NREM sleep and the EEG delta power, the latter being an index of sleep intensity (Krueger, 2008; Opp, 2005). In this regard, it is also noteworthy that TNF and IL-1β cause dysregulation of Clock genes with decreased expression of the period genes Per1, Per2 and Per3 and the PARbZip transcription factors including Dbp, Tef and Hlf (Cavadini et al., 2007).

The regulation of sleep has been studied intensively using sleep deprivation (SD). SD leads to increased expression of, e.g., immediate early genes/transcription factors, mitochondrial genes, genes involved in energy metabolism, and neurotransmitter transporters and receptors (Cirelli, 2009). Transcriptome profiling in inbred mouse strains showed that genetic background affects susceptibility to SD at the transcriptional level. When taking the genetic back- ground into account, expression of Homer1a associates with changes in homeostatic sleep need (Franken et al., 2001; Mackiewicz et al., 2008; Maret et al., 2007). Neurons expressing Homer1a also express early growth response 2 (Egr2/Krox20), Fos-like antigen 2 (Fosl2), prostaglandin-endoperoxide synthase 2 (Ptgs2), junctophilin 3 (Jph3), and neuronal pentraxin 2 (Nptx2) (Maret et al., 2007). Expression of these transcripts equally increases with sleep need. Homer1a mRNA increases during spontaneous and enforced periods of wakefulness (Huber et al., 2007a; Maret et al., 2007; Nelson et al., 2004). Mice with a deletion of Homer1a (Homer1a KO mice) show a reduced wakefulness with increased NREM sleep during the dark period (Naidoo et al., 2012; 2014). Previous studies of mice with CD40-induced sleep showed that Homer1a expression decreases during the second half of the dark period when mice show increased NREM sleep (Gast et al., 2013). In the cortex of CD40 mAb-treated mice the decline of Homer1a in the dark period was associated with a significant depression of Egr2, Nptx2, and Fosl2.

The effects of cytokines on sleep-wake behavior may involve communications of neurons with microglia. Cytokines may regulate intercellular interactions by promoting neurons to produce chemokines, which attract microglia to neuronal dendrites and synapses. In this study, we investigated whether TNF may lead to the expression of chemokines by neurons. The data presented here show that TNF activates neuronal production of CCL2, CCL7 and CXCL10 and of M-CSF/CSF-1. These chemokines have been well described to attract mononuclear phagocytes and to lead to the extension of microglial processes. By influencing synaptic strength and by secreting mediators such as glutamate and prostaglandins, TNF-stimulated microglia cells may play a pivotal role in the regulation of sleep. Our study on the effects of cytokines on neurons also addresses the question as to whether downregulation of Homer1a expression in CD40-activated mice is cytokine-mediated. Such effects would induce transitions between wakefulness and sleep. We found that HT22 cells and cortical neurons respond to TNF and IL-1β with increased Egr2 and Ptgs2 expression, but not with a downregulation of Homer1a. Thus in CD40 mAb-treated mice, the decrease of Homer1a expression in the dark period is not due to direct inhibitory effects of TNF and IL-1β on Homer1a transcription.

METHODS

Primary cortical neurons, HT22 cells and cytokine treatment

Neurons were isolated from the cerebral cortex of C57BL/6J mice gestational stage E14–E18 as previously described (Ogunshola et al., 2002). Dissected cortices were dissociated in Hank's buffered salt solution containing trypsin or papain and DNase I for 5 min at 37 °C. Neurons were seeded on poly-L-lysine coated petri dishes (3×10^6 cells per 100 mm dish) in Neurobasal medium containing B27 supplement (1x), AlbuMAX (0.25 g/ml), 1% sodium pyruvate, 100 U/ml penicillin-streptomycin and 1 mM L-glutamine (GIBCO, Invitrogen, AG, Switzerland). The cultures (purity 98%), were maintained for 17 days at normal atmosphere (21% O_2) in a humidified incubator at 37 °C. For analysis of cytokine expression, cells were treated on day 14–17

with TNF (Peprotech, London UK) or PBS control for 4-8 h. Cells and supernatants were harvested thereafter.

For expression of Homer1a, Egr2, Ptgs2 and Fosl2 primary cortical neurons were plated either on 35 mm dishes (at 1.5 x 10^6 cells; density = 1560 cells/mm^2) pre-coated with 0.1 mg/ml poly-L-lysine. Cultures were maintained in a humidified CO_2 incubator (5% CO_2, 37 °C) and half of the medium was changed once a week. After treatment of cortical neurons with cytokines or PBS control during 4 h, total RNA from cell cultures was extracted.

The mouse hippocampal neuronal cell line HT22 was obtained from David Schubert at the Salk Institute (La Jolla, CA). HT22 cells were plated in 12-well tissue cultures plates (100,000 cells per well) in DMEM with 10% FCS. Two days after plating, cultures were serum deprived for 1 h followed by treatment with TNF or IL-1β for 4 h before extraction of RNA.

RNA extraction and quantification

RNA was extracted using RNA easy mini kit (Qiagen). All RNA samples were DNase-treated and quantified on a NanoDrop ND-1000 spectrophotometer. To quantify the RNA expression level, 1 µg of RNA was reverse-transcribed in 20 µl using random hexamers and Superscript II reverse transcriptase (Invitrogen) according to standard procedures. The cDNA was diluted 10 times and 2 µl were amplified in a 10 µl TaqMan reaction on ABI PRISM HT 7900 detection system in technical triplicate. Cycler conditions were 50°C 2min, 95°C 10min and 45 cycles at 95°C 15s and 60 °C 1 min. Forward primer, reverse primer, and probe sequences are given in Table 1. The gene expression level was normalized to three reference genes (Rsp9, TBP, and EEF1a1) using Qbase software (Hellemans et al., 2007). The fold changes indicative of the relative gene expression are based on the mean of 3 biological replicates in relation to control samples. The statistical analysis was performed with a one-way ANOVA. To determine the changes in gene expression form the control group a Bonferroni post hoc test was performed.

Table 1: Primer sequences for RT-qPCR

Gene	Forward Primer 5'→3'	Reverse Primer 5'→3'	Probe 5'→3'
eEF1a1	CCTGGCAAGCCCATGTGT	TCATGTCACGAACAGCAAAGC	TGAGAGCTTCTCTGACTACCCTCCACTTGGT
Homer1a	GCATTGCCATTTCCACATAGG	ATGAACTTCCATATTTATCCACCTTACTT	ACACATTCAATTCAGCAATCATGA
RPS9	GACCAGGAGCTAAAGTTGATTGGA	TCTTGGCCAGGGTAAACTTGA	AAACCTCACGTTTGTTCCGGAGTCCATACT
TBP	TTGACCTAAAGACCATTGCACTTC	TTCTCATGATGACTGCAGCAAA	TGCAAGAAATGCTGAATATAATCCCAAGCG
Egr2	AGGCCCCTTTGACCAGATG	CTTCTCTCCAGTCATGTCAATGTTG	CGGAGTGGCGGGAGATGGCAT
Fosl2	AGTGATCAAGACCATCGGTACCA	CTCCGGATTCGACGCTTCT	CCGCAGAAGGAGAGATGAGCAGCTGT
Ptgs2	AGCGAGGACCTGGGTTCAC	TGTCCAGAGTTTCACCATAAATGTG	AGGACTGGGCCATGGAGTGGACTTAAA

Gene expression arrays

Cytokine and chemokine gene expression was tested using a cytokine gene array (Gene Expression System StellARay; Bar Harbor Biotechnology) according to the manufactures instructions. The results were analyzed with the ''Global Pattern Recognition'' (GPR) Tool from Bar Harbor Biotechnology. All array analyses were performed in triplicates.

Cytokine analysis

Cytokines were measured in supernatants of cell cultures by Luminex technology (BioRad) following the manufacturer's instructions.

RESULTS

Production of chemokines by TNF treated neurons

To assess whether in cortical neurons TNF leads to the production of a set of cytokines and chemokines, which act on mononuclear phagocytes rather than on lymphocytes, we studied the expression of cytokines using cytokine gene arrays. Our data show that TNF enhanced the expression of only a very limited number of cytokine genes. Out of 96 genes represented on the microarray only 7 transcripts were found to be upregulated more than 2-fold. TNF induced the expression of the chemokines Ccl2, Ccl5, Ccl7, Cxcl1, Cxcl5 and Cxcl10 (Table 2).

The only non-chemokine gene activated in cortical neurons was the macrophage colony stimulating factor (M-CSF/Csf1). We validated the gene array data obtained by quantifying the chemokine levels in the culture supernatants. In agreement with

the gene chip data, supernatants of TNF-treated cortical neurons showed increased concentrations of CCL2 (MCP1), CCL7 (MCP3) and CXCL10 (IP-10) (Table 3). CXCL1 was also induced by TNF, although the effect did not reach statistical significance. In contrast CCL5 (RANTES), and CXCL5 (LIX) were only increased at the mRNA level. M-CSF was detected in cortical neuron supernatants, but its production was not regulated by TNF.

Using gene array the cortical neurons treated with TNF were not found to show an increased expression of (i) proinflammatory cytokines (IL-1α/β, IL-6, GM-CSF/Csf1, G-CSF), (ii) cytokines involved in T cell development and activation such as IL-12, IL-17, IL-23, TGF-β, IFNγ, IL-4 and IL-5, and of (iii) cytokines that promote B cell lineage development, differentiation and activation (IL-6, BAFF and APRIL).

Table 3: Productions of cytokines by TNF treated neurons

Table 2: TNF upregulated cytokine genes in cortical neurons.

Gene	Gene symbol	Fold Change	P-value
Chemokine (c-x-c motif) ligand 10	Cxcl10	184	2.77^{-09}
Chemokine (c-c motif) ligand 2	Ccl2	108.4	7.96^{-07}
Chemokine (c-c motif) ligand 5	Ccl5	35.56	2.06^{-06}
Chemokine (c-c motif) ligand 7	Ccl7	19	4.34^{-08}
Chemokine (c-x-c motif) ligand 1	Cxcl1	11.72	4.48^{-07}
Chemokine (c-x-c motif) ligand 5	Cxcl5	5.78	6.33^{-05}
Macrophage colony stimulating factor	Csf1	2.77	0.0002

Cytokine	Control	TNF	Stimulation Index (SI)
CXCL10	100.6 ± 43.5	2237 ± 382*	22.3
CCL2	49 ± 19.5	332 ± 96*	6.8
CXCL1	9.2 ± 6.7	48.7 ± 35.7	5.2
CCL7	16.7 ± 0.2	48.5 ± 8.3*	2.9
M-CSF	184.6 ± 6.6	200.6 ± 65.4	1
CCL5	<10	<10	-
CXCL5	<10	<10	-

Expression of Homer1a, Egr2, Ptgs2 and Fosl2 in HT22 cells and cortical neurons

To determine whether TNF and IL-1β modulate the expression of Homer1a, Egr2, Ptgs2 and Fosl2, we treated the neuronal cell line HT22 with TNF for 4 h. As shown in Figure 1, TNF strikingly upregulated Ptgs2 and increased Egr2 in a dose dependent manner, both genes being most affected with a TNF concentration of 10 ng/ml. HT22 cells responded to the TNF-treatment with a slight increase of Homer1a, and did not react to the cytokine with a dose dependent increase of Fosl2 (above 2.0-fold). Besides TNF, also IL-1β is produced by CD40 mAb-treated macrophages and dendritic cells and has been implicated in increased NREM sleep and dysregulation of clock gene expression (Cavadini et al., 2007; Taraborrelli et al., 2011). Therefore, we examined whether the effect of TNF described here is a unique property of TNF or is shared by IL-1β. HT22 treatment with IL-1β only moderately increased Fosl2, but had no effect on Homer1a, Ptgs2 and Egr2 (Figure 2).

However when using primary cortical neurons and treating them with IL-1β the expression of Egr2 was enhanced (Figure 3). While the expression of Homer1a and Fosl2 remained unchanged irrespective of the dose of IL-1β added to the neuronal cultures, Ptgs2 mRNA was increased, the effect, however, not reaching statistical significance.

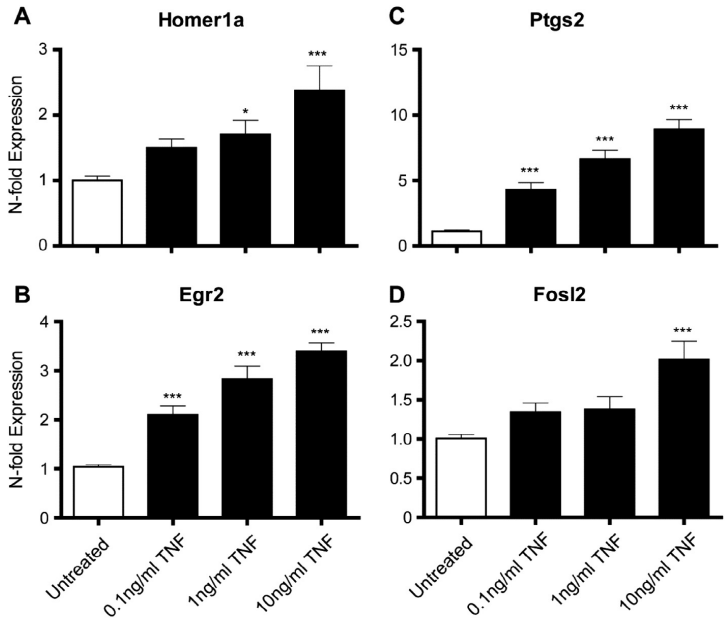

Figure 1: Treatment of HT22 cells with TNF induces the expression of Homer1a, Egr2, Ptgs2 and Fosl2. The hippocampal neuronal cell line HT22 is treated with TNF, (0.1 ng/ml; 1 ng/ml and 10

ng/ml) for 4 h (black bars). Compared to PBS control (white bars), treatment with TNF (10 ng/ml) increases the expression of Homer1a (A) Egr2 (B) Ptgs2 (C) and Fosl2 (D) by a factor of 2.4, 3.3, 8.1 and 2 respectively. Data of RT-qPCR assays of Homer1a, Egr2, Ptgs2 and Fosl2 expression shows the mean ± SEM of triplicates from four independent experiments. One-way ANOVA with Bonferroni post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

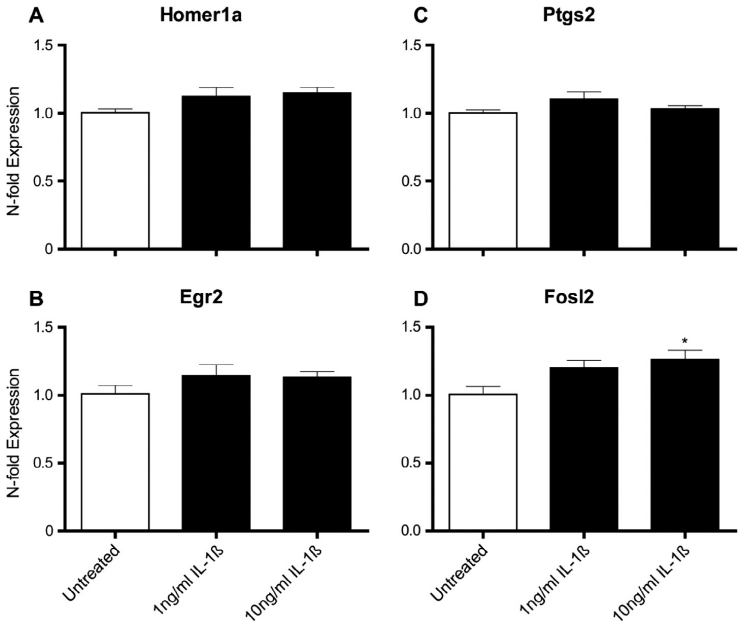

Figure 2: Treatment of HT22 cells with IL-1β slightly increased the expression of Fosl2, but not of Homer1a, Egr2 and Ptgs2. HT22 cells are treated with either 1 ng/ml IL-1β or with 10 ng/ml IL-1β for 4 h (black bars). Compared to PBS controls (white bars), none of the four genes tested was increased by IL-1β above 2-fold. Data of RT-qPCR assays of Homer1a, Egr2, Ptgs2 and Fosl2 expression shows the mean ± SEM of quadruplets for two independent experiments. One-way ANOVA with Bonferroni post hoc test. * $p < 0.05$.

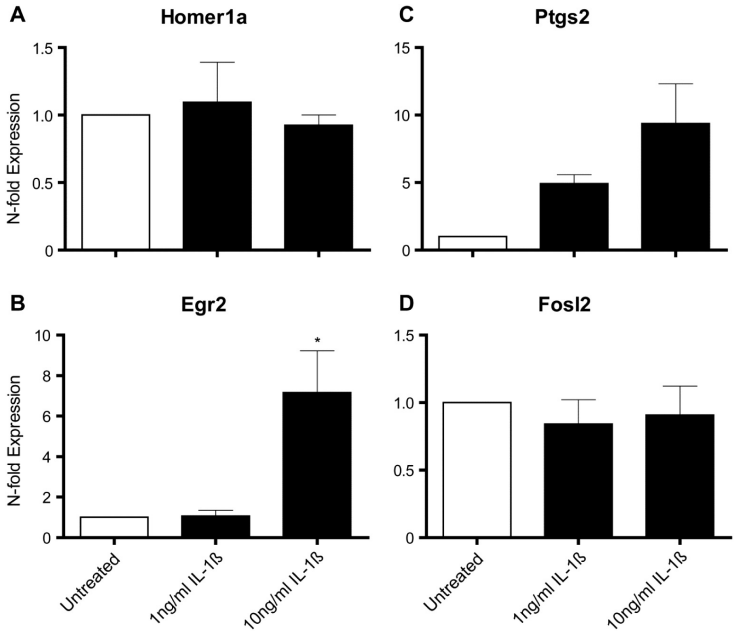

Figure 3: Treatment of primary cortical neurons with IL-1β enhances the expression of Egr2, but does not affect the expression of Homer1a and Fosl2. Cortical neurons were treated with either 1 ng/ml IL-1β or with 10 ng/ml IL-1β for 4 h (black bars). Compared to controls (white bars), IL-1β (10 ng/ml) highly upregulated Egr2. The stimulatory effects on Ptgs2 did not reach statistical significance. Data of RT-qPCR assays of Homer1a, Egr2, Ptgs2 and Fosl2 expression shows the mean ± SEM of quadruplets for two independent experiments. One-way ANOVA with Bonferroni post hoc test. * $p < 0.05$.

DISCUSSION

Cortical neurons, treated with TNF produce M-CSF/CSF-1 and the chemokines CCL2, CCL7 and CXCL10

The question arises as to how the production of somnogenic cytokines is induced in microglia cells in the physiological sleep wake cycles. A central role may be attributed to ATP. Neuronal activity has been found to be associated with synaptic co-release of ATP, which acts via P2x7 receptors on microglia to induce the release of TNF and IL-1β (Krueger et al., 2008). The pathway proposed requires direct interactions of neurons with microglia, the main producers of TNF in the brain. Cooperative interactions of the two types of cells may also be essential for microglia-mediated synaptic plasticity, including dendritic spine remodeling and elimination (Tremblay

and Majewska, 2011; Tremblay et al., 2011). Following visual stimulation microglia sample individual synapses more frequently compared with nonstimulated synapses (Tremblay et al., 2010). Thus, microglia may monitor the functional state of synapsis and contribute to plastic changes (Blank and Prinz, 2013). Synaptic strength is suggested to occur during wakefulness, downscaling of synaptic strength to basal level may play a fundamental role in sleep (Tononi and Cirelli, 2006). In the study presented here, we determine whether neurons produce factors that attract microglia. The results demonstrate that primary cortical neurons respond to TNF treatment with upregulated expression of the chemokine genes Ccl2, Ccl5, Ccl7, Cxcl1, Cxcl5 and Cxcl10. At the protein level, only CCL2, CCL7 and CXCL10 were upregulated. Reports on neurons as a possible source of chemokines are sparse, but indicate that they can make a contribution. For example, measles virus infection of primary hippocampal neurons induces CXCL10 and CCL5 production (Patterson et al., 2003). Scrapie-infected neurons mainly upregulate expression of CCL5, but also produce lower levels of other chemokines including CCL2 (Marella and Chabry, 2004). The chemokines identified here to be secreted by cortical neurons - CCL2 and CCL7 - attract cells of the monocyte-macrophage lineage. CCL2 and CCL7 bind to the chemokine receptor CCR2, which is expressed in microglia as well as on neurons (Banisadr et al., 2005; Boddeke et al., 1999). By attracting microglia, the neuronal production of CCL2 and CCL7 may play a pivotal role in neuron-microglia communication. CCL2 has also been shown to induce migration of neuroprogenitor cells and to direct neural progenitor cell migration following striatal cell loss (Gordon et al., 2009; Vrotsos and Sugaya, 2009). TNF treatment of cortical neurons increased the production of CXCL10. This chemokine is induced in neurons by infection with dengue virus and inhibits binding of the virus to cell surface heparan sulfate, a co-receptor for the virus (Ip and Liao, 2010). CXCL10 binds to CXCR3 on microglia and induces their migration (Rappert et al., 2004). Besides its chemotactic activity on mononuclear phagocytes, CXCL10 leads to recruitment of activated $CXCR3^+$ T cells, but not resting T cells, that do not express this receptor (Müller et al., 2010). Thus, regarding the physiologic rhythmic expression of TNF in the brain, the function of neuron-derived CXCL10 is confined to its activity on microglia rather than T cells. In this context, it is of note that TNF did not upregulate the expression of the lymphoid

chemokines CCL9, CCL21 and CXCL13, which are critical in the generation of adaptive immune responses (Lalor and Segal, 2010). Our cytokine gene array data also show that cortical neurons did not respond to TNF treatment with upregulated expression of proinflammatory cytokines (IL-1α/β, IL-6, TNF, GM-CSF, G-CSF), or of cytokines involved in regulation of lineage development of naive T cells into Th1, Th2, Th17 and regulatory T cells (IL-12, IL-23, TGF-β, IFNγ, IL-4 and IL-5). Moreover primary neurons failed to respond to TNF with expression of cytokines that promote B cell and plasma cell survival and expansion such as IL-6, B cell activating factor (Baff) and the proliferation-inducing ligand (APRIL). The lack of TNF-induced neuronal signals, which act on T and B cells may provide a safety strategy of the CNS aimed at prevention of immune priming and lymphocyte activation. Besides of chemokines we found neurons to secrete M-CSF/CSF-1, a growth factor for microglia cells. For neuronal-microglia communication it is interesting that M-CSF/CSF-1 leads to elongation of microglial processes (Smith et al., 2013). Beside of microglia, the CSF1 receptor is expressed on a small number of neurons in the hippocampus and cortex (Luo et al., 2013).

TNF and IL-1β do not downregulate Homer1a

Homer1a belongs to the plasticity-regulated genes that are upregulated as immediate-early genes during hippocampal long-term potentiation and epileptic seizures. Recent data suggest that Homer1a plays a pivotal role in the mechanisms that regulate sleep. Homer1a mRNA is increased in the dark phase in the somatosensory cortex of rats and is up-regulated with sleep loss, the effect being associated with sleep pressure and dependent on the strain of mice (Mackiewicz et al., 2008; Maret et al., 2007; Nelson et al., 2004). Moreover, Homer1a knockout mice fail to sustain long bouts of wakefulness (Naidoo et al., 2014). Our recent data on Homer1a expression in untreated mice are in agreement with the aforementioned studies. Homer1a increased with sleep need, lowest levels being observed when mice were resting (Gast et al., 2013). CD40 mAb treatment was found (1) to shorten the time of wakefulness during the dark phase, and (2) to lead from Zeitgeber ZT13 to ZT18 to an early decrease of Homer1a expression in the frontal cortex. Likewise, intraperitoneal injections of TNF at ZT9 decreased wakefulness within hours and lowered the expression of Homer1a

at ZT18. In the light of the failure of Homer1a knockout mice to keep wakefulness the decrease of Homer1a after CD40mAb or TNF injections may result from TNF or IL-1β mediated inhibition of Homer1a expression. Alternatively, the decrease of Homer1a may be the consequence of lower sleep pressure in the second phase of the dark period. Since investigations on regulation of Homer1a expression by proinflammatory cytokines are lacking, we treated cultured neurons with cytokines and assessed the expression of Homer1a. Our in vitro data show that neither TNF nor IL-1β suppressed the expression of Homer1a mRNA in HT22 neuronal cells and primary cortical neurons respectively. In fact the opposite was true. Homer1a mRNA was upregulated in HT22 cells treated with TNF, an effect not being seen with IL-1β. When taking the genetic background of different mouse strains into account, sleep loss-induced transcriptional changes involved only a limited number of genes (Maret et al., 2007). In addition to Homer1a also Egr2, Ptgs2 and Fosl2 were identified to consistently follow the altered expression of Homer1a during SD. As true for Homer1a, we found TNF and IL-1β to increase rather then to decrease the expression of these genes in HT22 and/or primary cortical neurons. In this respect it is of note that TNF has already been reported to enhance the expression of Egr1 and Fos-1 in cultures of vascular smooth muscle cells. These genes are like Egr2 and Fosl2 other members of the Egr and Fos family of transcriptional regulators respectively (Goetze et al., 2001). Moreover, Ptgs2, the gene encoding Cyclooxygenase-2 has been well described to be induced by proinflammatory cytokines including TNF and IL-1β (Cao et al., 2011). These data are in line with the results presented here showing TNF to upregulate Egr2 and Fosl2.

CONCLUSION

Our data on the response of neurons to TNF show that cortical neurons exposed to TNF produce chemokines (CCL2, CCL7, and CXCL10) and M-CSF/CSF-1. These factors have been described to attract microglia and to lead to extension of microglial processes. Thus, TNF may play a pivotal role in neuron-microglia communication, which allows synaptic plasticity and regulation of sleep. The increase of NREM sleep

induced by cytokines has been shown to be associated with decreased expression of Homer1a. This splice form of Homer1 has been shown recently to be required for maintenance of wakefulness. Our data using cultures of HT22 cells and primary cortical neurons show that TNF and IL-1β do not interfere with Homer1a expression in neurons in vitro. In the light of the complex molecular pathways involved in sleep regulation, the interpretation of these in vitro data must be done carefully. They do not support the hypothesis that the altered expression of Homer1a in cytokine-induced sleep is due to direct effects of cytokines on expression of Homer1a in neurons in vivo.

Author contributions

M.K., M.A.L., A.F.M., D.M., C.M. and O.O.O. designed and performed the experiments. A.F.M., L.S., M.T. and A.F. contributed to the concept of the study.

Competing interests

The authors have no competing interests to disclose.

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4. CONCLUSION

The term sickness behavior syndrome, describes the collective immune mediated adaptations of behavior and physiology that help to facilitate recovery from infection or injury. Among the most prevalent symptoms are increased sleep propensity in combination with sleep fragmentation, fatigue, reduced appetite, as well as lowered mood and social withdrawal as seen in affective disorders. These symptoms are a result of the burst production of cytokines, blood borne messenger molecules of the immune system, which signal to the brain about the present state of infection or injury. In chronic autoimmune or inflammatory diseases, such as rheumatoid arthritis or inflammatory bowel disease, continued production of cytokines results in SBS as comorbidity, severely affecting the quality of life of patients in addition to the underlying disease. The exact cellular processes that lead to the production of cytokines vary to a high degree and strongly depend on the type and stage of the disease as well as genetic factors. Nevertheless, unifying principles exist, and they may be exploited for the development of therapeutic strategies. For example, a common denominator of autoimmune diseases is the dependency on CD40-CD40L signaling and the subsequent production of TNF and other inflammatory mediators. Neutralizing antibodies and fusion proteins against TNF, like infliximab or etanercept, have become effective tools for the treatment of many inflammatory and autoimmune diseases, including psoriasis, rheumatoid arthritis and inflammatory bowel disease. Furthermore, SBS associated symptoms are significantly reduced with this approach. Not all patients respond to this type of treatment, however, and the risk of opportunistic infections, accompanying malignancies and high financial costs limit its applicability. Thus, understanding the mechanisms that govern the development of SBS could open new opportunities for therapeutic approaches, and may even shed light on the pathology of the underlying disease.

Mice have long been used as the primary model system for research into the mechanism of many human diseases due to a remarkably similar physiology. Given the obvious difference in brain organization and complexity however, studies in mice regarding behavior are limited in their design to address basic behavioral traits, and are generally difficult to translate to human psychological conditions. In our studies,

we have therefore focused on locomotor activity as simplified behavioral readout of sickness intensity. Additionally, cachexia, or wasting syndrome, the result of impaired food uptake and metabolism and a trademark of acute disease, can easily be determined via body weight measurements at different time points.

SBS is a very complex phenomenon, affecting many physiological processes including sleep, circadian rhythmicity, hormone balance and nutrient uptake. The work presented here therefore covers several aspects of sickness behavior, both from an immunological and from a neuroscientific perspective. Sickness behavior is commonly induced with injections of bacterial lipopolysaccharide or proinflammatory cytokines. These are excellent tools for the study of immediate behavioral effects of cytokines in a general setting, but they do not reflect the state of immune activation in autoimmune diseases. The latter are characterized by a sterile, exaggerated inflammatory response with high levels of CD40L produced by $CD4^+$ T cells. We therefore decided to use the recently established mouse model of CD40 ligation through monoclonal antibodies for most of our in vivo studies. Mimicking a prolonged and exaggerated CD40-CD40L signaling should be more representative of the situation seen in many autoimmune diseases.

CD40 is expressed by numerous cell types of different hematopoietic and non-hematopoietic origins, including endothelial cells. In our studies, we could show that the most important cell types in regards to the development of sickness behavior are tissue resident macrophages and monocytes, or monocyte-derived inflammatory cells. These cells produce large amounts of proinflammatory cytokines during the first two days after CD40 ligation, including TNF, IL-1β, IL18 and IFNγ. By neutralizing CSF1R, the receptor for the mitogen M-CSF, we were able to re-educate monocytes towards an excessive production of the anti-inflammatory cytokine IL-10, resulting in a near complete protection from the adverse behavioral and physiological effects of CD40 signaling. This occurs despite the high levels of TNF and IL18 that are present due to the ongoing inflammatory response. The nature of the CD40-induced inflammatory cells in terms of differentiation and polarization, as well as how their 'reprogramming' is achieved by neutralization of CSF1R, may be addressed in future studies using, for example, cell sorting and subsequent gene expression analysis.

As we could show, the drop in locomotor activity induced by CD40 ligation is mainly due to an increase in the amount and intensity of NREM sleep. This effect seems to be dependent on TNF signaling, as mice treated with the TNF blocker etanercept are resistant to the sleep inducing effect of anti-CD40 antibodies. This confirms the result of a previous study using TNFR1 knockout mice (Taraborrelli et al., 2011). Thus, the protective effect of IL-10 is therefore most likely due to an inhibition of the somnogenic effects of TNF and other cytokines.

As to how this signal is transmitted to the brain is still a matter of debate. Several possible routes exists, all of which are most likely involved to some degree. Cytokines in the peritoneal cavity can be sensed by the vagus nerve, while cytokines in the blood assert their effect through circumventricular organs, where they diffuse into parts of the brain parenchyma. The rest of the brain, as well as the spinal chord, is shielded from blood borne agents by the blood brain barrier, a structural component of the brain vasculature that prevents circulating molecules from crossing the endothelial wall. Thus, cytokines, chemokines and antibodies such as those against CD40 and CSF1R used in our studies, are prevented from reaching their target structures in the CNS. However, it has recently been shown that during CD40-mediated inflammation the function of the BBB is compromised, allowing cytokines and other agents to directly reach their targets in the brain, including neurons, astrocytes, and microglia (Konsman et al., 2008; Laflamme and Rivest, 1999; Qin et al., 2007). Anti-CSF1R treatment may therefore have the same IL-10-inducing effect on microglia as it has on monocytes, with the local production of IL-10 in the brain being dependent on a disruption of the BBB.

Another open question is how the neutralization of CSF1R induces IL-10 production, a hallmark of alternatively activated macrophages. At first glance, these results seem to be in contradiction to what is currently known about macrophage biology. Studies with glioblastoma, for example, have shown that neutralization of CSF1R has the exact opposite effect on tumor-associated macrophages (TAM) then what we see in our experiments. In this model, tumor growth is reduced after treatment with a CSF1R-inhibitor due to the surviving TAMs losing their M2-like, tumor-promoting phenotype (Pyonteck et al., 2013). A explanation lies in the fact that long term treatment with antibodies against CSF1R results in the accumulation of CSF-1, likely due to a lack of receptor-mediated endocytosis. CD40 activation induces massive cell proliferation.

Thus, while the concentration of the CSF1R antibody may be sufficient for steady state conditions, it may not be high enough to block ligand-receptor interaction during CD40-mediated inflammation. The highly elevated levels of CSF-1 may therefore produce a net signal that polarizes macrophages towards the observed anti-inflammatory phenotype. The situation may be similar in the brain despite a lack of cell proliferation. A disrupted BBB would allow the entry of excess CSF-1, while CSF1R antibodies would mostly be sequestered on peripheral myeloid cells.

In addition to sleep, cytokines have a profound effect on the expression of clock genes. For example, Cavadini and colleagues demonstrated that TNF reduces the binding activity of CLOCK:BMAL1 complexes to the regulatory E-box element, resulting in the strongly decreased expression of several core clock and clock controlled genes, including Period 1-3, Dbp, Hlf and Tef both in synchronized fibroblasts and in mouse liver (Cavadini et al., 2007). Moreover, we identify a novel mechanism for the effect of TNF and TGF-β on the expression of clock genes. Cold inducible RNA-binding protein (CIRBP) has been described as an important regulatory molecule driving high amplitude expression of clock genes by increasing mRNA stability of its target genes (Morf et al., 2012). Here we show that TNF- and TGF-β-mediated downregulation of Cirbp expression in neuronal cells results in a reduced expression of Period 3, Dbp, Hlf and Tef.

These results blend nicely with our data gained from patients with Alzheimer's disease (AD). In this study, we report that TGF-β found in cerebrospinal fluid of AD patients may downregulate clock genes in the brain of these patients, contributing to the highly prevalent sleep disturbances seen in AD.

Rhythmic clock gene expression forms the basis of circadian patterns in metabolism and behavior. The specific effect of clock gene downregulation on behavior or sleep homeostasis is difficult to discern, however, and may be limited to a passive role. The 'flattening' of the clock gene amplitude throughout the day in CD40 mAb-treated mice may abolish the influence of the circadian clock on behavioral and physiological processes, such as the consolidation of wakefulness during the subjective day, or regulation of immune system responsiveness. In other words, downregulation of the clock would induce a shift in the balance of stimuli towards those favoring a sustained sickness behavior and inflammatory response.

A more direct link between gene expression and behavior can be drawn in the case of Homer1a, the short, inhibitory isoform of the gene *Homer1*. Together, they regulate the stability of GluR5 receptors in the postsynaptic membrane and seem to play a role in synaptic plasticity. While *Homer1a* is usually expressed in a strictly circadian fashion, it has been identified together with several other genes as being associated with sleep loss. During prolonged periods of wakefulness, *Homer1a* expression in the frontal cortex is increased with the same rate as delta power, and declines during recovery sleep. *Homer1a* knockout animals show an increased propensity to fall asleep during their activity period, which shows *Homer1a* to have an important function in the consolidation of wakefulness. Consequently, in mice treated with CD40 mAb, *Homer1a* is downregulated during the course of the day, enabling the transition to sleep during times mice are normally awake and active.

In a similar fashion to *Homer1a*, *Per2* has been associated with sleep homeostasis, thus integrating the molecular clock with the homeostatic regulation of sleep. However, neither our *in vitro* studies with TNF-treated neuronal cell lines, nor our *in vivo* studies using the CD40 ligation model have demonstrated a significant effect of cytokines on *Per2* expression in cortical neurons. A possible role for this clock gene in sickness behavior remains to be determined.

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6. ACKNOWLEDGEMENTS

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And of course, Martin Lopez, my 'brother in arms', and one of the most diligent and honest people I have ever met. I depended more on his help and advice than I would like to admit so a big thank you is in order at this point.

There are too many people who have helped me in one way or another to name them all. Needless to say, I owe the most to all the members of our lab, both present and former who have all contributed countless times to the planning, execution, and analysis of my many experiments.

Calling research work 'not always fun' would be a mild understatement. So when science lets you down, you should have someone to pick you up and get you going again. In that regard, it is still amazing to me how many friends I have made during these past four years, and I hope I will carry many of them with me into the next chapter. Sooner or later I am going to let it roll with my mates Tom and Andy, chill out with Isaak and Kay, have a good single malt with Tvisha, and have a hours long conversation with Kathrin. Thank you all for being as awesome as you are.

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7. APPENDIX

7.1 Author Contributions

The following experiments were done by Andreas Müller (in collaboration were indicated):

3.1 Neutralization of colony-stimulating factor 1 receptor prevents sickness behavior syndrome by reprogramming inflammatory monocytes to produce IL-10

Fig. 1 A-D
Fig. 2 A-C
Fig. 4 A-D
Fig. S1

In collaboration with Laura Strauss:
Fig. 3 A-C

3.2 CD40 activation induces NREM sleep and modulates genes associated with sleep homeostasis

Fig. 3 A-F
Fig. 6
Fig. 7
Table 1

3.3 Tumor necrosis factor and transforming growth factor beta regulate clock genes by controlling the expression of the cold inducible RNA-binding protein (CIRBP)

In collaboration with Martin Lopez:
Fig. 10
Suppl. Table 1

3.4 Transforming growth factor-beta inhibits the expression of clock genes

In collaboration with Martin Lopez:
Fig. 3
Fig. 4

3.5 Cytokine-induced sleep: Neurons respond to TNF with production of chemokines and increased production of *Homer1a* in vitro

In collaboration with Maureen Karrer and Martin Lopez:
Fig. 1 A-D
Fig. 2 A-D
Fig. 3 A-D

7.2 Curriculum Vitae

Andreas Müller, PhD

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Scientific Education

01/11 – 03/15	**University of Zurich, Switzerland** Doctoral studies in Immunology and Neuroscience
07/02 – 04/10	**Technical University of Munich (TUM), Germany** Diploma in Biology Major: Genetics, Virology, Human Physiology

Scientific and Other Work Experience

03/15 – present	**Postdoctoral Researcher at the University of Zurich, Inst. of Experimental Immunology, Research Group Prof. Adriano Fontana, Switzerland**
01/11 – 03/15	**Doctoral studies at the University of Zurich, Inst. of Experimental Immunology, Research Group Prof. Adriano Fontana, Switzerland** Project title: Role of macrophages in cytokine-mediated sleep induction
04/10 – 05/10	**Technician at the Center of Life and Food Sciences Weihenstephan, Dept. of Plant Developmental Biology, Research Group Prof. Kay Schneitz, Germany**
07/09 – 04/10	**Diploma Thesis at the Center of Life and Food Sciences Weihenstephan, Dept. of Plant Developmental Biology, Research Group Prof. Kay Schneitz, Germany** Project title: Interaction studies between the AGC kinases UNICORN, UNICORN-LIKE and PDK1
07/08 – 12/08	**Technician at the Helmholtz Center Munich, Inst. of Developmental Biology, Research Group Dr. Chichung Lie, Germany**
04/06 – 07/06	**Laboratory Internship at the Helmholtz Center Munich, Inst. of Developmental Biology, Research Group Dr. Chichung Lie, Germany** Project Title: Generation of retroviral shRNA-vectors to study the function of STAT3 in adult neurogenesis

10/05 – 01/06	**Laboratory Internship at the Helmholtz Center Munich, Inst. of Experimental Genetics, Research Group Prof. Martin Hrabě de Angelis, Germany** Project Title: Role of the cytoplasmic domain of Delta-like1 in mouse embryonic development
11/01 – 07/02	Military Service as a motor mechanic in the German Armed Forces in Lenggries, Germany

Skills

Technical Skills	Basic laboratory techniques (qRT-PCR, molecular cloning, cell culture, western blot, ELISA); flow cytometry; use of Luminex technology; histology - IHC/ICC; analysis of circadian gene expression patterns; gene array Extensive training in the use and handling of laboratory animals, with specialization in mice. Work experience includes: injection techniques; sampling of tissues; measuring of EEG patterns with implanted electrodes; assessment of locomotor activity.
Teaching Experience	Tutorship in practical course "Medical Immunology", University of Zurich, Inst. of Exp. Immunology, 2013 Tutorship in course "Molecular and Classical Genetics", University of Zurich, Inst. of Molecular Life Sciences, 2013 Supervision of intern, University of Zurich, Inst. of Exp. Immunology, 2011

Language Skills	German	Native speaker
	English	Proficient user
	Dutch	Basic user
	Japanese	Basic user (JLPT Level 4, as of 2009)

Informatics Skills	Highly experienced in the use of data analysis tools, including PRISM, Clocklab, Qbase, Flowjo; graphic design: Adobe Illustrator
Other Skills	Organization of collaborative projects; financial planning; basic knowledge in Swiss patent law

Publications

Müller A.F., Strauss L., Greter M., Gast H., Becher B., Fontana A., *Blocking of MCSF1R leads to Macrophage polarization towards an anti-inflammatory phenotype.* Brain, Behavior, and Immunity. 2015 Article in Press

Gast H., **Müller A.F.**, Lopez M., Meier D., Huber R., Dechent F., Prinz M., Emmenegger Y., Franken P., Birchler T., Fontana A. *CD40 activation induces NREM sleep and modulates genes associated with sleep homeostasis.* Brain, Behavior, and Immunity. 2013 Jan;27(1):133–44.

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Supplementary Information and References

Personal Interests	Music	Playing guitar, songwriting
	Outdoor recreation	Mountain-biking, skiing
	Sports	Wing Tsun Kung Fu, weight training

References

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